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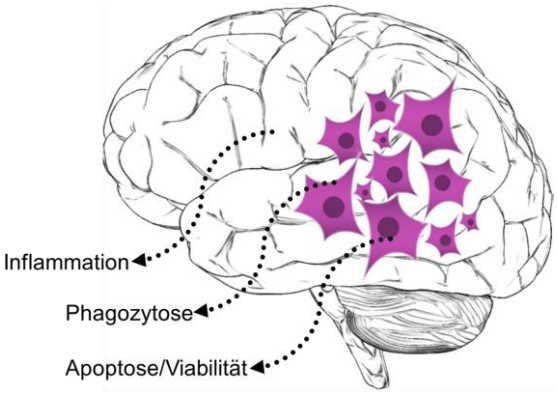
Geschäftsführender Direktor: Prof. Dr. Dr. h.c. Wolfgang H. Oertel
des Fachbereichs Medizin der Philipps-Universität Marburg

Modulation mikroglialer Zellen in der Alzheimer-assoziierten Neuroinflammation



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Maike Gold
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Anmerkung:

In dieser Promotion wurde von der Möglichkeit der kumulativen Promotion (nach der „Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fachbereiche und des Medizinischen Fachbereichs für seine mathematisch-naturwissenschaftlichen Fächer der Philipps-Universität Marburg vom 15.7.2009 (§9)) Gebrauch gemacht. Demnach sind eingereichte oder veröffentlichte Publikationen als Dissertationsleistung anzuerkennen. Die vorliegende Arbeit besteht aus einer gemeinsamen Einleitung, der Zusammenfassung der Ergebnisse sowie einer gemeinsamen Diskussion. Am Ende sind die publizierten Originalarbeiten angefügt.

Originalarbeiten

1. **Gold, M.**, D. Mengel, S. Roskam, R. Dodel und J. P. Bach (2013). Mechanisms of action of naturally occurring antibodies against beta-amyloid on microglia. J Neuroinflammation. 10: 5 10.1186/1742-2094-10-5.

2. Mengel, D., S. Roskam, F. Neff, K. Balakrishnan, O. Deuster, **M. Gold**, W. H. Oertel, M. Bacher, J. P. Bach und R. Dodel (2013). Naturally occurring autoantibodies interfere with beta-amyloid metabolism and improve cognition in a transgenic mouse model of Alzheimer's disease 24 h after single treatment. Transl Psychiatry 3, e236 10.1038/tp.2012.151.

3. Dolga, A. M., T. Letsche, **M. Gold**, N. Doti, M. Bacher, N. Chiamvimonvat, R. Dodel und C. Culmsee (2012). Activation of KCNN3/SK3/K(Ca)2.3 channels attenuates enhanced calcium influx and inflammatory cytokine production in activated microglia. Glia 60(12): 2050-64.

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1. Einleitung

1.1 Die Alzheimer-Krankheit

Die Alzheimer-Krankheit (englisch: *Alzheimer's Disease*, AD) ist die häufigste neurodegenerative Erkrankung des zentralen Nervensystems. Weltweit waren im Jahr 2006 26,6 Millionen Menschen betroffen und bis zum Jahr 2050 wird sich diese Zahl vermutlich vervierfacht haben (Brookmeyer et al. 2007). Neben der familiären Form von AD, die ungefähr 5-10 % der Fälle ausmacht, gibt es auch eine sporadische Form der Erkrankung. Die Ursache für die progressive Demenzerkrankung, die durch einen ausgeprägten Neuronenverlust gekennzeichnet ist, ist noch nicht vollständig aufgeklärt. Histopathologisch lassen sich allerdings zwei prägnante Merkmale feststellen, die für die Krankheit pathognomonisch sind: die extrazellulare Ablagerung von Amyloid-Plaques und die intrazelluläre Bildung von Neurofibrillenbündeln (Braak und Braak 1990). Da ich mich in den Fragestellungen dieser Arbeit auf die Aβ-Toxizität beschränke, werde ich im Folgenden nicht speziell auf die pathologischen Mechanismen des Tau-Proteins eingehen. Die neuesten Erkenntnisse zur Tau-Pathologie bei AD wurden von Mandelkow und Mandelkow in einer exzellenten Übersichtsarbeit zusammengefasst (Mandelkow und Mandelkow 2012).

Amyloid-Plaques bestehen hauptsächlich aus aggregierten Amyloid-β (Aβ)-Peptiden. Es kommen unterschiedliche Aβ-Spezies vor, die je nach Anzahl der verknüpften Aminosäuren $Aβ_{1-x}$ benannt werden. In den Plaques findet man hauptsächlich $Aβ_{1-40}$ und $Aβ_{1-42}$, wobei sich gezeigt hat, dass $Aβ_{1-42}$ schneller aggregiert als $Aβ_{1-40}$ (Jarrett et al. 1993). Die Zusammenlagerung von Aβ-Monomeren zu Amyloid-Plaques läuft über die Bildung kleinerer Oligomere, größerer Oligomere und der Bildung von Fibrillen. Vor allem die löslichen oligomeren Formen scheinen neurotoxisch zu sein (Hartley et al. 1999; Cleary et al. 2005). Es konnte gezeigt werden, dass injizierte lösliche Oligomere die Kognition von Ratten stark beeinträchtigen. Die löslichen Oligomere wirken sich dabei über verschiedene MAP (englisch: *mitogen-activated protein*)-Kinasen und die Aktivierung metabotroper Glutamatrezeptoren negativ auf die Langzeitpotenzierung aus (Wang et al. 2004). Des Weiteren kann die Aktivierung von Caspasen und damit verbundene Apoptose durch die Behandlung mit synthetischen Aβ-Oligomeren *in vitro* nachgewiesen werden (Benilova et al. 2012).

Die Bildung von Aβ läuft über das Amyloid-Vorläuferprotein (englisch: *Amyloid-Precursor-Protein*, APP), wobei das APP prinzipiell auf zwei Wegen prozessiert werden kann (Abb. 1, Seite 2). Es gibt den amyloidogenen Weg, bei dem zunächst durch eine β-

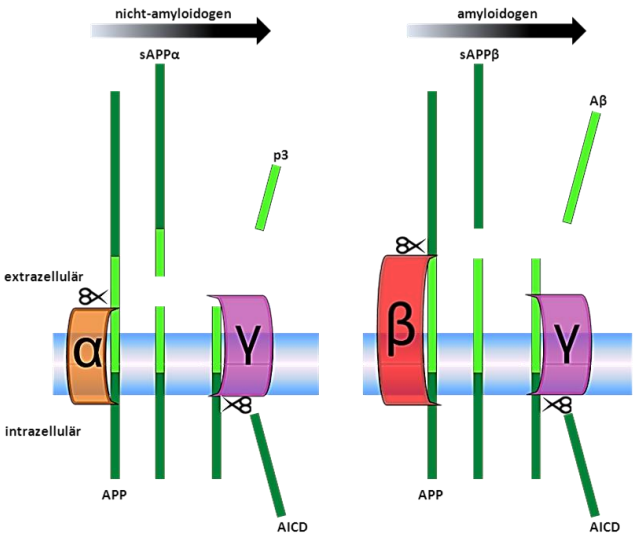

Abb.1 Bildung von Aβ aus dem Amyloid-Vorläuferprotein
[modifiziert nach (O'Brien und Wong 2011)]

Sekretase das lösliche Fragment sAPPβ abgespalten wird und anschließend durch γ-Sekretasen Aβ freigesetzt wird. Beim nicht-amyloidogenen Weg wird in einem ersten Schritt durch α-Sekretasen lösliches sAPPα abgespalten und in einem zweiten Schritt durch γ-Sekretasen das kleine Fragment p3 freigesetzt. Da die Schnittstelle der α-Sekretasen in der Aβ-Sequenz liegt, werden im Rahmen des nicht-amyloidogenen Wegs keine Aβ-Spezies gebildet. Bei beiden Wegen wird zudem eine intrazelluläre Domäne des APP (AICD) abgespalten (O'Brien und Wong 2011).

Über die Funktion der APP-Spaltprodukte ist nur wenig bekannt. sAPPα wird eine neuroprotektive Rolle zugeschrieben, während sAPPβ die Aktivierung von Caspase-6 begünstigen soll (Chow et al. 2010). Auch die Funktion von AICD wird kontrovers diskutiert. *In vitro* Studien konnten zeigen, dass AICD durch die Bindung an das Adapter-Protein Fe65 und die Rekrutierung der Histon-Deacetylase TIP60 an der Aktivierung der Transkription verschiedener Zielgene beteiligt ist (Kimberly et al. 2001). Unter den Zielgenen dieses Komplexes sind unter anderem p53, Glykogensynthase-Kinase 3β sowie verschiedene Cycline, über deren Regulation Einfluss auf den Zelltod genommen werden kann (Pardossi-Piquard und Checler 2012).

Es existieren Formen der familiären AD bei denen, durch die Begünstigung des amyloidogenen Abbauwegs des APP, die Entstehung von AD gefördert wird. So sind zum Beispiel Punktmutationen im APP oder Mutationen in den Presenilin-Genen, die Bestandteil des katalytischen Zentrums der γ-Sekretase sind, bekannt (Bertram und Tanzi 2008).

Neben dem starken Neuronenverlust und dem Auftauchen der Plaques und Fibrillen kommt es mit dem Fortschreiten der Erkrankung zunehmend zu Entzündungserscheinungen im Gehirn. Post-mortem Analysen von Alzheimer-Patienten zeigen aktivierte Mikrogliazellen, die hauptsächlich um Amyloid-Plaques herum lokalisiert sind (El Khoury et al. 1996).

1.2 Neuroinflammation, die Rolle mikroglialer Zellen

Mikrogliazellen machen je nach Gehirnregion bis zu 20 % der Gliazellen und damit einen großen Teil der Gehirnmasse aus (Lawson et al. 1990). Auf Grund ihrer immunomodulatorischen Funktion werden sie auch als residente Makrophagen des Gehirns bezeichnet. Neben dem hämatopoetischen Ursprung verfügen sie über ein sehr ähnliches Rezeptor-Profil wie Makrophagen in der Peripherie (Kettenmann et al. 2011). Man unterscheidet zwischen ruhenden Mikrogliazellen und durch Infektionen, traumatische Stimuli oder neurodegenerative Erkrankungen konvertierte aktivierte Mikrogliazellen. Aktivierte Mikrogliazellen nehmen eine amöboide Form an, migrieren an den Ort des Insults (Stence et al. 2001) und produzieren viele verschiedene pro-inflammatorische Mediatoren wie Zytokine, reaktive Sauerstoffspezies und Stickstoffmonoxid (NO) (Rupalla et al. 1998). Diese tragen in der Regel zu einer Beseitigung des Pathogens bei. Für die Aktivierung der Mikrogliazellen entscheidend ist hierbei auch ein Anstieg der intrazellulären Kalziumspiegel (Hoffmann et al. 2003). Zusätzlich haben Mikrogliazellen die Fähigkeit zur Phagozytose. Sie sind also maßgeblich daran beteiligt, die Homöostase des Gehirns aufrecht zu erhalten, indem sie Moleküle, Debris und apoptotische Zellen aufnehmen und beseitigen. Über verschiedene Aufnahmewege sind Mikrogliazellen dazu in der Lage, Aβ aus dem Extrazellularraum aufzunehmen (Mohamed und Posse de Chaves 2010). Da Aβ-Oligomere neurotoxisch sind, spielen Mikrogliazellen in diesem Fall eine neuroprotektive Rolle. Die Bandbreite an Reaktionen aktivierter Mikroglia umfasst auch die Produktion sogenannter Neurotrophine (Elkabes et al. 1996). Neurotrophine sind kleine Moleküle, die sich ebenfalls protektiv auf das Überleben von Neuronen auswirken (Ghosh et al. 1994).

Der Rolle von Mikrogliazellen bei AD wurde in den letzten Jahren immer mehr Aufmerksamkeit zuteil. Neben des toxischen Effekts auf Neurone haben Aβ-Oligomere eben auch einen toxischen Effekt auf Mikrogliazellen (Pan et al. 2011) oder können Mikrogliazellen aktivieren. Sowohl *in vitro*, nach Behandlung mit Aβ, als auch *in vivo*, in post-mortem Gehirnen von Alzheimer-Patienten, konnten erhöhte Level an pro-inflammatorischen Zytokinen und NO festgestellt werden (Ii et al. 1996; Pyo et al. 1998; Morimoto et al. 2011). Aβ-Oligomere scheinen hier, genauso wie die Bestandteile von bakteriellen Zellwänden, Signalkaskaden über Toll-like-Rezeptoren zu aktivieren und so zur Sekretion der pro-inflammatorischen Substanzen zu führen (Walter et al. 2007). Die initial neuroprotektive Wirkung von Mikrogliazellen durch die Aufnahme von Aβ und die Sekretion neurotropher Substanzen, scheint mit anhaltender Aktivierung und der chronischen Sekretion pro-inflammatorischer Botenstoffe zunehmend geringer zu werden (Akiyama et al. 2000). Auch die Wirkung pro-inflammatorischer Zytokine wird im

Moment noch kontrovers diskutiert. Dabei scheint es eine Frage des richtigen Equilibriums zu sein, ob pro-inflammatorische Zytokine eher eine positive oder negative Rolle bei AD spielen (Wee Yong 2010). Ein geringer vorübergehender Anstieg pro-inflammtorischer Zytokine kann sich positiv auswirken, wohingegen die Induktion eines exzessiven und lang-anhaltenden Anstiegs zu chronischer Neuroinflammation und Neurodegeneration führt. Wichtig ist nach derzeitigem Kenntnisstand allerdings vor allem die Wiederherstellung des richtigen Equilibriums (Ji et al. 2011).
Die Unterbindung der mit AD einhergehenden Neuroinflammation bietet, neben dem Eingriff in die Prozessierung des APP mit Sekretase-Inhibitoren, eine weitere Möglichkeit der Intervention. Die Behandlung von Alzheimer-Patienten mit nicht-steroidalen Antirheumatika hatte bisher keinen positiven Effekt auf die Progression der Erkrankung (Heneka et al. 2011). Die Behandlung eines kleineren Patientenkollektivs mit Etanercept, einem Antikörper gegen das pro-inflammatorische Zytokin Tumornekrosefaktor-α (TNF-α), hatte rasch positive Auswirkungen auf die Kognition (Tobinick 2009). Allerdings sind weitere Studien hierzu zur Validierung der Ergebnisse vonnöten. Eine weitere Therapieoption ist die aktive oder passive Immunisierung. Durch die Markierung der Amyloid-Plaques mit Antikörpern wird unter anderem deren Abbau durch Antigen-Antikörper-Komplexbildung und dadurch die Möglichkeit der Fcγ-Rezeptor-abhängigen Phagozytose durch Mikrogliazellen begünstigt (Delrieu et al. 2012).

1.3 Natürlich vorkommende Autoantikörper gegen Aβ

In den letzten 15 Jahren wurden Untersuchungen zur aktiven und passiven Immunisierung als Therapie von AD durchgeführt. Rationale hierfür ist unter anderem die Förderung der Beseitigung von Amyloid-Plaques. *In vivo* in transgenen Mäusen wurden mit aktiver Immunisierung vielversprechende Ergebnisse erzielt, jedoch kam es bei der Anwendung beim Menschen mit der Substanz AN1792, die aus fibrilliertem $A\beta_{1-42}$ und dem Immunstimulans QS-21 bestand, zu Meningoenzephalitiden (Gilman et al. 2005). Die Anwendung humanisierter, monoklonaler Antikörper (Bapineuzumab, Solanezumab) als passive Immunisierung im Menschen in Phase III klinischen Studien zeigte zwar eine Reduktion der Amyloid-Plaques, jedoch keine deutliche Verbesserung der Kognition bei der Gruppe von Patienten mit leichter bis moderater Alzheimer-Krankheit (Grundman et al. 2013). Eine weitere Möglichkeit der passiven Immunisierung bietet die Anwendung von intravenösen Immunglobulinen (IVIg) als Therapie bei AD.
IVIg werden aus einem Serumpool von mehreren tausend gesunden Spendern gewonnen und spiegeln die gesamte IgG-Diversität der einzelnen Spender wider. IVIg haben ein sehr breites Anwendungsspektrum und werden bereits seit längerer Zeit

erfolgreich in der Therapie von Autoimmunerkrankungen und bei immundefizienten Patienten eingesetzt. Untersuchungen zur Anwendung bei AD sind im Moment Gegenstand einer Phase III klinischen Studie (www.clinicaltrials.gov/ct2/show/NCT01736579). Inakzeptable Nebenwirkungen wie bei der aktiven Immunisierung sind in einer Phase II Studie an Patienten mit leichter bis moderater Alzheimer-Krankheit nicht beobachtet worden, jedoch lassen sich auch noch keine exakten Aussagen bezüglich der Wirksamkeit auf die Kognition treffen (Dodel et al. 2013).

Bestandteil von IVIg sind auch sogenannte natürlich vorkommende Autoantikörper (englisch: *Naturally occurring autoantibodies,* nAbs) gegen Aβ (Dodel et al. 2002). nAbs machen etwa zwei Drittel des humanen Antikörperpools aus und deren mannigfaltige Funktionen und Wirkweise sind noch nicht aufgeklärt (Shoenfeld et al. 2006). Vermutlich werden nAbs stetig und unabhängig von einem externen Stimulus von sogenannten B1-Lymphozyten gebildet (Hayakawa et al. 1985). Eine lokale, intrathekale Bildung der Antikörper im Gehirn ist bei intakter Blut-Hirn-Schranke eher unwahrscheinlich. Im Gegensatz zu B2-Lymphozyten, die in Lymphknoten des gesamten Körpers außer des zentralen Nervensystems vorkommen, sind B1-Lymphozyten hauptsächlich im Peritonealraum angesiedelt (Hayakawa et al. 1985). Abhängig von der Expression des CXC-Chemokin Ligand 13 sind sie in der Lage aus dem Peritonealraum weg und wieder zurück zu migrieren (Ansel et al. 2002). Bei der Bildung der Antikörper bedienen sich B1-Lymphozyten eines eingeschränkten V(D)J-Gen-Segment Repertoires (Meffre und Salmon 2007) und sind Teil des angeborenen Immunsystems (Haas et al. 2005). Diese Bildung geschieht unabhängig von einem zusätzlichen T-Lymphozyten Stimulus (Hayakawa et al. 1985). Dadurch erfahren B1-Lymphozyten weder einen Ig-Klassenwechsel noch expandieren sie klonal.

Im zentralen Nervensystem spielen nAbs eine wichtige Rolle bei der Aufrechterhaltung der Homöostase, indem sie unter anderem zur Beseitigung von Protein-Aggregaten beitragen (Gold et al. 2012). nAbs gegen α-Synuclein (Papachroni et al. 2007), Tau-Protein (Rosenmann et al. 2006), Prion-Protein (Wei et al. 2012) und Aβ (Du et al. 2001) im Menschen wurden schon beschrieben. Interessanterweise wurden bei Alzheimer-Patienten sowohl im Serum als auch in der Zerebrospinalflüssigkeit (englisch: *cerebrospinal fluid*, CSF) eine geringere Konzentration an nAbs-Aβ festgestellt (Du et al. 2001; Weksler et al. 2002; Britschgi et al. 2009). Positive Effekte von nAbs-Aβ wurden bereits *in vitro* an mit Aβ behandelten neuronalen Zelllinien und murinen primären Neuronen, sowie *in vivo* an transgenen Alzheimer-Mäusen gezeigt (Dodel et al. 2011). Auch die Existenz katalytischer Autoantikörper gegen Aβ wurde beschrieben (Taguchi et al. 2008). Diese Antikörper vom IgM-Typ sind in der Lage Aβ *in vitro* zu spalten. Welche Rolle nAbs-Aβ allerdings im Hinblick auf mikrogliale Zellen spielen, ist bislang

nicht untersucht worden. Eine Korrelation zwischen der Ansammlung körpereigener IgGs um Aβ-Plaques und der Anzahl neuritischer Plaques sowie phagozytierender Mikrogliazellen bei Alzheimer-Patienten wurde bereits festgestellt (Kellner et al. 2009) und Experimente mit IVIg zeigen einen erheblichen Einfluss auf die phagozytotische Aktivität und Viabilität der mikroglialen Zelllinie BV-2 und auf murine primäre Mikrogliazellen (Istrin et al. 2006; Magga et al. 2010).
Es ist daher sinnvoll, auch für die Untersuchung des Einflusses der natürlich vorkommenden Autoantikörper auf Mikrogliazellen nicht mit IVIg, sondern mit der mutmaßlich aktiven Substanz im Hinblick auf AD nämlich nAbs-Aβ, zu arbeiten.

1.4 Die Rolle der SK-Kanäle bei Neuroinflammation

Neben dem Einfluss von nAbs-Aβ auf die Neuroinflammation ist es auch Gegenstand dieser Arbeit, mögliche Modulationen der SK (englisch: *small conductance*)-Kanal Physiologie von Mikrogliazellen hinsichtlich deren Aktivierungszustands zu untersuchen. SK-Kanäle bilden eine Unterfamilie der Kalzium-aktivierten Kaliumkanäle. Durch die Erhöhung intrazellulärer Kalziumspiegel wird der Kanal selektiv permeabel für Kalium. Die Sensitivität für Kalzium wird hierbei über das assoziierte Ca^{2+}-bindende Protein Calmodulin übertragen. SK-Kanäle haben Calmodulin konstitutiv an ihrem C-Terminus gebunden. Durch die Bindung von Ca^{2+} an Calmodulin erfährt der SK-Kanal eine Konformationsänderung und ein Kaliumausstrom wird möglich (Xia et al. 1998).
Die Rolle der SK-Kanäle in Neuronen ist weitestgehend bekannt: durch die selektive Permeabilität für Kalium sind sie maßgeblich an der Bildung der Hyperpolarisation von Aktionspotentialen in Neuronen beteiligt (Hosseini et al. 2001). Dadurch nehmen SK-Kanäle indirekt Einfluss auf intrazelluläre Kalziumlevel, dendritische Erregbarkeit, synaptische Weiterleitung und synaptische Plastizität (Faber 2009). Auch in Mikrogliazellen werden verschiedene SK-Kanäle vom Typ SK1, SK2 und SK3 exprimiert (Schlichter et al. 2010). Die Rolle der SK-Kanäle in Mikrogliazellen ist bislang nur wenig untersucht worden. CyPPA (Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine) ist ein spezifischer SK2- und SK3-Kanal-Aktivator (Hougaard et al. 2007) und erlaubt dadurch die gezielte Untersuchung dieser Kanäle. Mit der Anwendung von CyPPA in Glutamat-behandelten primären Neuronen konnte das intrazelluläre Kalziumlevel gesenkt und die Zellen vor dem Untergang geschützt werden (Dolga et al. 2011). Interessanterweise gibt es eine Untersuchung bei der intrazelluläre Kalziumlevel von kultivierten Mikrogliazellen post-mortem in Alzheimer-Patienten und nicht dementen Kontrollen verglichen wurden (McLarnon et al. 2005). Mikrogliazellen von Alzheimer-Patienten zeigten signifikant erhöhte Kalzium-Basislevel. Da also durch die Aktivierung

von SK-Kanälen in Neuronen intrazelluläre Kalziumlevel gesenkt werden konnten und Kalziumlevel auch bei der Aktivierung von Mikrogliazellen eine große Rolle spielen, soll im Folgenden die Rolle der SK-Kanäle bei der Aktivierung der Mikrogliazellen untersucht werden.

2. Ergebnisse

Eigene Anteile an den Publikationen:

1. **Gold, M.**, D. Mengel, S. Roskam, R. Dodel und J. P. Bach (2013). Mechanisms of action of naturally occurring antibodies against beta-amyloid on microglia. J Neuroinflammation 10: 5 10.1186/1742-2094-10-5 **(Gold et al. 2013)**.

   Ich habe das Projekt gemeinsam mit Dr. Jan-Philipp Bach und Prof. Dr. Richard Dodel geplant und alle Experimente, bis auf die Tierversuche in Abb.3, selbstständig durchgeführt und die Ergebnisse ausgewertet. Das Manuskript habe ich gemeinsam mit Dr. Jan-Philipp Bach und Prof. Dr. Richard Dodel entworfen und geschrieben.

2. Mengel, D., S. Roskam, F. Neff, K. Balakrishnan, O. Deuster, **M. Gold**, W. H. Oertel, M. Bacher, J. P. Bach und R. Dodel (2013). Naturally occurring autoantibodies interfere with beta-amyloid metabolism and improve cognition in a transgenic mouse model of Alzheimer's disease 24 h after single treatment. Transl Psychiatry 3, e236 10.1038/tp.2012.151 **(Mengel et al. 2013)**.

   Diese Studie ist Teil der Doktorarbeit von cand. med. David Mengel. Ich habe die Experimente zu den Abb. 1d, 1e und 2b geplant und durchgeführt, sowie am Manuskript mitgearbeitet.

3. Dolga, A. M., T. Letsche, **M. Gold**, N. Doti, M. Bacher, N. Chiamvimonvat, R. Dodel und C. Culmsee (2012). Activation of KCNN3/SK3/K(Ca)2.3 channels attenuates enhanced calcium influx and inflammatory cytokine production in activated microglia. Glia 60(12): 2050-64 **(Dolga et al. 2012)**.

   Diese Originalarbeit entstand in Zusammenarbeit mit der Arbeitsgruppe von Prof. Dr. Culmsee (Institut für Pharmakologie und pharmazeutische Chemie, Philipps-Universität Marburg) und ist Teil der Doktorarbeit von cand. med. Till Letsche. Ich habe zur Durchführung und Auswertung der Zytokin ELISAs in den Abb. 2C, 2D, 6A, 6B, 6D, 6E, 6Fund 6G beigetragen. Des Weiteren war ich an der Anfertigung des Manuskripts beteiligt.

2.1 Der Einfluss von nAbs-Aβ auf Viabilität, Zytokinausschüttung und Phagozytose auf Mikrogliazellen *in vitro* hat einen positiven Effekt auf primäre Neurone.

Der Einfluss von nAbs-Aβ auf Aβ-behandelte Neurone wurde bereits ausführlich untersucht und die positive Wirkung sowohl *in vitro* an Zelllinien und primären Neuronen als auch *in vivo* in transgenen Alzheimer-Mäusen ist hinlänglich bekannt (Du et al. 2003; Dodel et al. 2011). In der ersten Originalarbeit sollte die Auswirkung von nAbs-Aβ auf Aβ-behandelte Mikrogliazellen untersucht werden. Als Negativkontrolle wurde in allen Experimenten der Durchfluss der affinitätschromatographischen Aufreinigung, also IVIg ohne nAbs-Aβ, eingesetzt und als ft-Aβ (englisch: *flow-through*) gekennzeichnet.

Die Viabilität von Mikrogliazellen wurde durch die Behandlung mit oligomerisiertem $Aβ_{1-42}$ im MTT (-3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) Assay auf annähernd 50 % reduziert (Gold et al., 2013, Abb. 1). Die Vorbehandlung mit nAbs-Aβ hatte hierauf keinen Einfluss. Die Behandlung mit nAbs-Aβ an sich hatte keinen Effekt auf die Viabilität der Mikrogliazellen.

Da nAbs-Aβ keinen Einfluss auf die Viabilität der Mikrogliazellen zeigten, wurden als nächstes intrazelluläre Stress-Signalwege untersucht. Oligomerisiertes $Aβ_{1-42}$ führte bereits nach kurzer Zeit zu einer Phosphorylierung und damit zu einer Aktivierung der MAP-Kinase p38 (Gold et al. 2013, Abb. 2A). Durch die gleichzeitige Behandlung mit nAbs-Aβ kam es zu einem 10-fachen Anstieg im Vergleich zu unbehandelten Zellen (Gold et al. 2013, Abb. 2B). Die Behandlung mit nAbs-Aβ an sich zeigte nur eine sehr geringe Phosphorylierung von p38. Die Ergebnisse der pro-inflammatorischen Zytokine gehen mit diesen Beobachtungen einher: Die Co-Stimulation von $Aβ_{1-42}$ und nAbs-Aβ führte zu einem signifikanten Anstieg der pro-inflammatorischen Zytokine Interleukin-6 (IL-6) und TNF-α (Gold et al. 2013, Abb. 2C und 2D). Auch bei der Behandlung mit nAbs-Aβ allein war ein leichter Anstieg von TNF-α mittels ELISA (englisch: *Enzyme-linked immunosorbent assay*) messbar.

Als residente Makrophagen des Gehirns haben Mikrogliazellen auch die Fähigkeit zur Phagozytose. Takata *et al*. haben bereits den Einfluss monoklonaler Aβ Antikörper auf die Phagozytose von Aβ durch Mikrogliazellen untersucht und konnten eine Steigerung der Phagozytoserate von Aβ feststellen (Takata et al. 2007). Um den Effekt von nAbs-Aβ auf die Phagozytose von $Aβ_{1-42}$ in Mikrogliazellen zu untersuchen, wurden zwei verschiedene Methoden angewandt. Mit Hilfe von Fluoresceinisothiocyanat (FITC)-markiertem $Aβ_{1-42}$ wurde die Phagozytose mittels Durchflusszytometrie untersucht. Hierbei konnte eine Verschiebung der mittleren Fluoreszenz-Intensität des aufgenommenen FITC-Aβ durch die Behandlung mit nAbs-Aβ erreicht werden (Gold et al. 2013,

Abb. 4A). Die Fluoreszenz-Intensität stieg signifikant um das doppelte an (Gold et al. 2013, Abb. 4B). In einer zweiten Versuchsreihe wurde die Aufnahme von Aβ in Mikrogliazelllysaten mit einem monoklonalen Aβ Antikörper im Western-Blot nachgewiesen (Gold et al. 2013, Abb. 4C). Die Banden-Intensitäten wurden densitometrisch erfasst und die Ergebnisse von drei unabhängigen Experimenten zusammengefasst (Gold et al. 2013, Abb. 4D). Auch hier hatte sich die Menge des aufgenommenen Aβ der Mikrogliazellen durch die Zugabe von nAbs-Aβ signifikant verdoppelt. ft-Aβ zeigte keinen Effekt auf die Aufnahme von oligomerisiertem $Aβ_{1-42}$.

Zusammenfassend zeigen diese Ergebnisse den Effekt von nAbs-Aβ auf Aβ-behandelte Mikrogliazellen. Während es keinen Effekt auf die Viabilität gibt, sehen wir einen Anstieg der Ausschüttung pro-inflammatorischer Zytokine und eine deutliche Steigerung der Phagozytose von Aβ durch Mikrogliazellen. Um einen möglichen indirekten Effekt von Mikrogliazellen auf Neurone zu untersuchen, wurde die Viabilität von primären Neuronen, die in konditionierten Mikrogliazellkulturüberständen kultiviert wurden, mittels MTT Assay gemessen. Es zeigte sich, dass die Neurone, die mit konditionierten Überständen von Mikrogliazellen, die sowohl mit nAbs-Aβ als auch mit Aβ behandelt wurden, ein signifikant höheres Überleben zeigten als solche, die nur mit Aβ behandelt wurden (Gold et al. 2013, Abb. 5). Kulturüberstände von Aβ-behandelten Zellen führten zu einer Viabilitätsreduktion primärer Neurone auf 67 %. Durch die zusätzliche Behandlung mit nAbs-Aβ reduzierte sich die Viabilität auf nur noch 77 %. Kulturüberstände von Mikrogliazellen die mit ft-Aβ und Aβ behandelt wurden, zeigten keinen positiven Effekt auf die Viabilität primärer Neurone.

2.2 nAbs-Aβ modulieren die Zytokinausschüttung *in vivo* im Gehirn von Tg2576 Mäusen.

Nachdem ein Anstieg pro-inflammatorischer Zytokine *in vitro* beobachtet wurde, haben wir in der ersten und zweiten Originalarbeit auch den Effekt von nAbs-Aβ *in vivo* in Tg2576 Mäusen untersucht. Tg2576 Mäuse exprimieren eine mutierte Form des APP ($APP_{SWE\ (695(K670N,\ M671L}$) unter der Kontrolle eines Hamster Prion Promotors (Hsiao et al. 1996). Durch die doppelte Mutation im APP bilden die Mäuse neuropathologische und verhaltenstypische Merkmale der AD aus. In der ersten Originalarbeit wurden 20-22 Monate alte Tiere einmalig mit nAbs-Aβ behandelt und die Zytokinkonzentrationen im Gehirn nach 24 Stunden mittels ELISA bestimmt. Im Vergleich zu Wildtyp-Tieren hatten unbehandelte Tg2576 Mäuse ein signifikant erhöhtes Basislevel der pro-inflammatorischen Zytokine Interleukin-1β (IL-1β), Interferon-γ, TNF-α und IL-6. Durch die Behandlung mit nAbs-Aβ wurde die Zytokinausschüttung nicht beeinflusst (Gold et

al. 2013, Abb. 3A-D). Für die zweite Originalarbeit wurden ältere Tiere (27-30 Monate alt) mit nAbs-Aβ für 24 Stunden behandelt (Mengel et al 2013, Abb. 3a-d). Erstaunlicherweise konnten in diesem fortgeschrittenen Stadium sämtliche pro-inflammatorischen Zytokine durch die Behandlung mit nAbs-Aβ auf das Zytokin-Niveau der Wildtyp-Tiere abgesenkt werden.
nAbs-Aβ scheinen zumindest bei kurzer Applikationsdauer keinen, beziehungsweise einen positiven Einfluss auf die Neuroinflammation der Alzheimer-Mäuse zu haben.

2.3 nAbs-Aβ beeinflussen den Aggregationszustand von Aβ in Tg2576 Mäusen und *in vitro* und verbessern die synaptische Plastizität und Kognition in Tg2576 Mäusen.

Neben der Untersuchung der Zytokinlevel von behandelten und unbehandelten Tieren wurde in der zweiten Originalarbeit auch der Einfluss von nAbs-Aβ auf Aβ-Level in Gehirn und CSF untersucht. Die Behandlung mit nAbs-Aβ führte zu einer starken Zunahme von Monomeren im Gehirn (Mengel et al. 2013, Abb.1d), wobei die Gesamtmenge an Aβ in Gehirn und CSF nicht beeinflusst wurde. Es handelt sich also lediglich um eine Verschiebung der Aggregationsformen von Aβ hin zur monomeren Form. Um zwischen den einzelnen Aβ-Spezies unterscheiden zu können, wurde ein spezielles Harnstoff-Gel verwendet (Klafki et al. 1996; Wiltfang et al. 1997). Es fiel auf, dass hauptsächlich $Aβ_{1-40}$-, wenig $Aβ_{1-42}$- und kaum $Aβ_{1-38}$-Monomere in den nAbs-Aβ-behandelten Tieren nachweisbar sind. Erstaunlicherweise ließ sich auch ein Einfluss von nAbs-Aβ *in vitro* auf den Oligomerisierungsprozess darstellen. Bei der Zugabe von nAbs-Aβ zum Oligomerisierungsprozess von $Aβ_{1-42}$ ließ sich eine deutliche Abnahme von größeren Oligomeren und eine damit einhergehende Zunahme von Monomeren sowie Di- und Trimeren beobachten (Mengel et al. 2013, Abb. 2a). nAbs-Aβ haben demnach eine Auswirkung auf die *de novo* Synthese von Oligomeren größerer Ordnung. Eine Co-Inkubation mit nAbs-Aβ – anschließend zum Oligomerisierungsprozess – hatte hingegen keine Wirkung mehr auf die Aggregationsformen *in vitro* (Mengel et al. 2013, Abb. 2b).
Neben der neuroinflammatorischen Modulation *in vivo*, sowie der Einflussnahme auf die Aggregationsformen von Aβ hin zu weniger toxischen Formen, hatte die einmalige nAbs-Aβ Applikation in Tg2576 Mäusen auch positive Auswirkung auf die synaptische Plastizität sowie auf die Kognition der Tiere. Um die synaptische Plastizität auszuwerten, wurden Neurone des visuellen Kortex immmunhistochemisch angefärbt und sowohl Axonlänge gemessen, als auch die synaptischen Kontakte ausgezählt. Durch die Behandlung mit nAbs-Aβ verdoppelte sich beides signifikant im Vergleich zu unbehan-

delten Tg2576 Mäusen (Mengel et al. 2013, Abb.5). Um Veränderungen in der Kognition zu untersuchen, wurde ein Objekt-Erkennungstest durchgeführt. Hierbei zeigte sich, dass Wildtyp-Mäuse und nAbs-Aβ-behandelte Tg2576 Mäuse signifikant mehr Zeit mit einem Objekt interagierten, wenn diesem Objekt ein neuer Platz zugewiesen wurde.

2.4 Die Aktivierung der Ca^{2+}-aktivierten Kaliumkanäle SK2 und SK3 reduziert die Aktivierung von Mikrogliazellen.

Eine weitere Möglichkeit Neuroinflammation zu modulieren, bietet sich durch den Eingriff in den intrazellulären Kalziumspiegel. Kalzium fungiert in Mikrogliazellen als Aktivierungskontrolle und eine Erhöhung intrazellulärer Kalziumspiegel ist für die Induktion pro-inflammatorischer Zytokine essentiell (Hoffmann et al. 2003).

Als Messparameter für eine mögliche Mikrogliazellaktivierung wurden in der dritten Originalarbeit NO, pro-inflammatorische Zytokine, morphologische Veränderung über Impedanz-abhängige Signale und die Proliferation primärer Mikrogliazellen mittels MTT Assay bestimmt. Die Behandlung mit bakteriellem Lipopolysaccharid (LPS) führte in Abhängigkeit von Zeit und Konzentration zu einem ansteigenden Impedanzsignal, das mit einer Änderung der Zellmorphologie einherging (Dolga et al. 2012, Abb. 1A und 1B). Durch die zusätzliche Behandlung mit CyPPA, einem SK-Kanal-Aktivator, konnte das Impedanzsignal nach einem kurzen Anstieg nahezu auf Ausgangsniveau zurückgebracht werden (Dolga et al. 2012, Abb. 1G). Zusätzlich konnte durch die Behandlung mit CyPPA, die durch LPS induzierte NO-Produktion sowie die Ausschüttung der pro-inflammatorischen Zytokine IL-6 und TNF-α, signifikant erniedrigt werden (Dolga et al. 2012, Abb. 2B, 2C und 2D). Die intrazelluläre Signaltransduktion scheint hierbei über die Phosphorylierung der MAP-Kinase p44/42 vermittelt zu werden (Dolga et al. 2012, Abb. 2E). Diese Effekte waren durch eine Prä-Stimulation mit CyPPA 24 Stunden vor LPS-Stimulation noch sehr viel ausgeprägter (Dolga et al. 2012, Abb. 3A-D) und benötigten extrazelluläres Ca^{2+} (Dolga et al. 2012, Abb. 4A-D).

Um den zeitlichen Verlauf intrazellulärer Kalziumspiegel nach LPS-Behandlung und Co-Stimulation mit CyPPA zu beobachten, wurden Messungen mit dem Ca^{2+}-Indikator Fura-2-acetoxymethyl Ester gemacht (Dolga et al. 2012, Abb. 4G und 4H). CyPPA scheint den durch LPS hervorgerufenen Anstieg von intrazellulärem Ca^{2+} in Mikrogliazellen signifikant reduzieren zu können. Um herauszufinden, welcher SK-Kanal Subtyp für die Modulation der Aktivierung der Mikrogliazellen verantwortlich ist, wurden die Experimente zusätzlich mit verschiedenen spezifischen inhibitorischen Peptiden der jeweiligen SK-Kanäle durchgeführt. Lediglich ein für den SK3-Kanal spezifisches inhibitorisches Peptid konnte den Effekt von CyPPA auf die Zellmorphologie antagonisieren

(Dolga et al. 2012, Abb. 5D). Dieser Einfluss schlug sich auch auf Ebene der NO-Produktion und der Zytokinausschüttung von IL-6 nieder (Dolga et al. 2012, Abb. 6C und 6E). Die Reduktion der TNF-α Ausschüttung durch die Behandlung von CyPPA konnte durch das spezifische SK3-Kanal inhibitorische Peptid nicht reversibel gemacht werden (Dolga et al. 2012, Abb. 6D), was für eine SK3-Kanal unabhängige Modulation spricht. Um den Einfluss des intrazellulären Kalziumspiegels weiter zu untersuchen, wurde dem Kulturmedium der Ca^{2+}-Chelator Ethylendiamintetraacetat (EDTA) zugesetzt. Mit steigender EDTA-Konzentration nahm die IL-6 Konzentration in den Kulturüberständen ab (Dolga et al. 2012, Abb. 6G), wohingegen die TNF-α Ausschüttung davon nicht beeinflusst wurde (Dolga et al. 2012, Abb. 6F).

3. Diskussion

In dieser kumulativen Arbeit wurden zwei mögliche Interventionsmöglichkeiten für die Alzheimer-assoziierte Neuroinflammation untersucht.

3.1 Der Einfluss von nAbs-Aβ auf Mikrogliazellen *in vitro* und *in vivo* in der Alzheimer-assoziierten Neuroinflammation

Der positive Effekt von nAbs-Aβ auf Aβ-behandelte Neurone und die Tatsache, dass IVIg eine potentiell positive Wirkung auf die Kognition von AD-Patienten hat, ist bereits publiziert worden (Relkin et al. 2009). Zudem haben Alzheimer-Patienten reduzierte nAbs-Aβ-Spiegel im Vergleich zu Gesunden der gleichen Altersgruppe (Du et al. 2001; Weksler et al. 2002). In dieser Arbeit wurde der Effekt von nAbs-Aβ auf Aβ-behandelte Mikrogliazellen *in vitro* in Bezug auf Zytokin-Sekretion und Aβ-Phagozytose untersucht. Weiter wurde untersucht, ob sich diese Veränderungen positiv auf Neurone auswirken. *In vivo* im transgenen Mausmodell wurden Entzündungsparameter nach der einmaligen Gabe von nAbs-Aβ gemessen.

Obwohl sich die Viabiliät von geschädigten Neuronen mit der Administration von nAbs-Aβ deutlich verbessert (Du et al. 2003; Dodel et al. 2011), konnte dieser Effekt in unserem System in primären Mikrogliazellen nicht beobachtet werden (Gold et al. 2013). Es ist hierbei zu beachten, dass im Vergleich zu den hier dargestellten Experimenten an Mikrogliazellen andere Aβ-Spezies, unterschiedliche Protokolle zur Oligomerisierung/Fibrillation und ein deutlich höheres oder niedrigeres Verhältnis der Molarität auf den Zellen von Aβ zu nAbs-Aβ verwendet wurden. Positiv zu werten ist, dass die Behandlung mit nAbs-Aβ an sich keine toxische Wirkung auf mikrogliale Zellen aufweist – eine wichtige Erkenntnis im Hinblick auf eine mögliche therapeutische Anwendung.

Eine Aβ-induzierte Inflammation der Zellen wurde durch die Behandlung mit nAbs-Aβ verstärkt. Dies konnte sowohl auf Ebene der MAP-Kinase p38, als auch an der sezernierten Menge pro-inflammatorischer Zytokine gezeigt werden (Gold et al. 2013). Interessanterweise führt eine einmalige Injektion von nAbs-Aβ in der transgenen Mauslinie Tg2576 nicht zu erhöhten Zytokinlevel im Gehirn. Ganz im Gegenteil scheint es je nach Behandlungsalter der Tiere sogar zu einer Reduktion sämtlicher pro-inflammatorischer Zytokine im Maushirn zu kommen (Gold et al. 2013; Mengel et al. 2013). Die Diskrepanz zwischen den *in vitro* und *in vivo* Ergebnissen bezüglich der Zytokinlevel kann man mit der Diversität der Zelltypen *in vivo* begründen – neben den *in vitro* untersuchten mikroglialen Zellen kommen im Gehirn unter anderem auch Astrozyten vor, die an

der Modulation der Neuroinflammation beteiligt sind. In einer *in vitro* Studie von von Berhardi *et al.* wurde gezeigt, dass eine durch Aβ induzierte Entzündungsreaktion von Mikrogliazellen hervorgerufen und durch die Anwesenheit von Astrozyten abgemildert wird (von Bernhardi und Eugenin 2004). Es ist schon lange bekannt, dass Astrozyten das Neurotrophin NGF (englisch: *nerve growth factor*) sezernieren. Durch die Stimulation mit den pro-inflammatorischen Zytokinen IL-1β und TNF-α kann diese Menge innerhalb von 48 Stunden um das vier- bis sechs-fache gesteigert werden (Gadient et al. 1990). Eventuell könnten auch zur Charakterisierung der Wirkmechanismen von nAbs-Aβ gemischte Gliazellkulturen Aufschluss geben. Ein weiterer Aspekt, der bei der Interpretation der Daten berücksichtigt werden muss, ist der kurze Behandlungszeitraum der Mäuse von nur 24 Stunden. Zwar ist bekannt, dass nAbs-Aβ die Blut-Hirnschranke überqueren können (Bacher et al. 2009), jedoch kann eine deutliche Anreicherung der nAbs-Aβ erst nach mehr als 48 Stunden beobachtet werden. Gegenstand weiterer Untersuchungen müssen folglich einerseits eine länger andauernde repetitive Behandlung mit nAbs-Aβ mit anschließender Quantifizierung pro-inflammatorischer Zytokine und andererseits die Quantifizierung pro-inflammtorischer Zytokine zu einem späteren Zeitpunkt sein. Ein möglicher Wirkmechanismus von nAbs-Aβ auf den Aktivierungszustand mikroglialer Zellen könnte hier die Inhibition der *de novo* Oligomer-Synthese durch nAbs-Aβ sein (Mengel et al. 2013). Da die Aβ-Oligomer-Last im Gehirn mit der Behandlung mit nAbs-Aβ abnimmt, könnten dadurch auch direkt weniger Mikrogliazellen aktiviert sein und die Zytokinlevel gesenkt werden.

Wie in der *in vitro* Studie gezeigt wird, führt die Administration von nAbs-Aβ auch dazu, dass deutlich mehr Aβ aus dem Extrazellularraum phagozytiert wird (Gold et al. 2012). Diese Tatsache könnte neben der inhibitorischen Wirkung auf die *de novo* Synthese der Oligomere zusätzlich dazu beitragen, dass die Oligomer-Last *in vivo* abnimmt. Die gesteigerte Phagozytoserate von Aβ lässt sich vermutlich darauf zurückführen, dass nAbs-Aβ mit Aβ-Oligomeren Antigen-Antikörper-Komplexe bilden. Antigen-Antikörper-Komplexe werden in der Regel Fc-Rezeptor-vermittelt in Mikrogliazellen aufgenommen (Koenigsknecht-Talboo und Landreth 2005). Zu beachten ist allerdings, dass in unserem experimentellen Versuchsansatz humane nAbs-Aβ im murinen Modellorganismus verwendet wurden. Zwar sind einige humane und murine Fcγ-Rezeptoren in weiten Teilen homolog und die Bindung von humanem Fcγ-Rezeptor an murine Antikörper ist bekannt (Gessner et al. 1998), jedoch ist nicht bekannt ob humane Antikörper an murine Fcγ-Rezeptoren binden können. Kürzlich wurde von Smith *et al.* ein Mausmodell vorgestellt, in welchem alle murinen Fcγ-Rezeptoren durch humane Fcγ-Rezeptoren ersetzt wurden (Smith et al. 2012). Es wäre eine Möglichkeit nAbs-Aβ in diesem Kontext auszutesten, um den möglichen Einfluss der Spezies auszuschließen. Der Einfluss

des monoklonalen murinen Antikörpers 6E10 auf die Phagozytose von Aβ wurde bereits untersucht (Takata et al. 2007) – auch mit diesem Antikörper konnte eine gesteigerte Phagozytoserate von Aβ beobachtet werden. Eine Immunreaktion der Mäuse gegen humanes IgG als unerwünschte Nebenwirkung in unserem Versuchsaufbau scheint sich in Grenzen zu halten. Mäuse, die 28 Tage lang mit humanen IgGs behandelt wurden, zeigten nur leicht erhöhte Antikörpertiter und keine pathologischen oder histopathologischen Veränderungen an sämtlichen Organsystemen (Dodel et al. 2011). Inwieweit sich nun die beobachteten Änderungen, die nAbs-Aβ auf Aβ-behandelte Mikrogliazellen verursachen, positiv auf Neurone auswirken, war Gegenstand weiterer Untersuchungen. Hierfür wurden primäre murine Neurone mit den Überständen von behandelten Mikrogliazellen stimuliert, und anschließend die Viabilität gemessen. Interessanterweise konnte mit den Überständen der gleichzeitigen Behandlung von nAbs-Aβ und Aβ der Mikrogliazellen, eine höhere Viabilität der Neurone festgestellt werden, als bei der Behandlung der Mikrogliazellen mit Aβ alleine (Gold et al. 2012). Es wurde eine Steigerung der Viabilität um 10 % festgestellt. Mit ft-Aβ konnte keine signifikante Veränderung der Viabilität gemessen werden. Die Behandlung mit nAbs-Aβ von Aβ-behandelten Mikrogliazellen führt also zu einer Milieuänderung, die sich positiv auf die Viabilität der Neurone auswirkt. Zwar wurde eine erhöhte Phagozytoserate festgestellt und die Annahme, dass eventuell eine verringerte Gesamtmenge an Aβ-Oligomeren für diesen Effekt verantwortlich ist, liegt nahe. Messungen des Aβ-Gehalts in den Überständen der Mikrogliazellen mittels ELISA konnten diesen Verdacht allerdings nicht stützen (Daten nicht gezeigt). Durchaus möglich ist, dass die von den Mikrogliazellen phagozytierte Menge an Aβ zu gering ist, um tatsächlich messbare Unterschiede *in vitro* feststellen zu können. Mit der Zugabe der nAbs-Aβ könnte auch die Aβ-Konformation im Medium zu einer weniger toxischen hin verändert werden. Bei *in vitro* Versuchen mit der Zugabe von nAbs-Aβ nach dem Oligomerisierungsprozess, wie in der zweiten Originalarbeit gezeigt wurde, konnten allerdings keine Unterschiede in den Konformationen von Aβ im Western-Blot festgestellt werden (Mengel et al. 2013), was diese Erklärung wiederum eher unwahrscheinlich macht. In einer Arbeit von Nakajima *et al.* wurde gezeigt, dass Mikrogliazellen nach der Aktvierung mit LPS Neurotrophine sezernieren (Nakajima et al. 2001). Eventuell könnten durch die gleichzeitige Behandlung mit nAbs-Aβ die gleichen Signalwege aktiviert werden. Untersuchungen der Überstände auf BDNF (englisch: *Brain-derived neurotrophic factor*) hin zeigten allerdings keinen Anstieg. Weitere Neurotrophine wie NT (Neurotrophin)-3, NT-4/5 oder GDNF (englisch: *Glia-derived neurotrophic factor*) könnten eine Rolle spielen und die Überstände der Mikrogliazellen sollten auch auf diese Substanzen hin getestet werden.

Zusammenfassend kann man feststellen, dass nAbs-Aβ innerhalb kürzester Zeit einen positiven Effekt *in vivo* haben, was sich zusätzlich zu der bereits bekannten positiven Auswirkung auf Neurone, auch auf die Beeinflussung der Physiologie der Mikrogliazellen zurückführen lässt. Die Applikation von nAbs-Aβ ruft, zumindest über einen kurzen Zeitraum, keine Neuroinflammation hervor. Viel mehr sind nAbs-Aβ vor allem in sehr alten Tieren in der Lage eine schon bestehende Neuroinflammation einzudämmen.

3.2 Der Einfluss des SK-Kanal-Aktivators CyPPA auf die Aktivierung von Mikrogliazellen *in vitro*

Eine weitere Möglichkeit des Eingriffs in die Neuroinflammation wurde in der dritten Originalarbeit untersucht (Dolga et al. 2012). Die Anwendung eines SK-Kanal-Aktivators zeigte, Ca^{2+}-vermittelt, einen Einfluss auf den Aktivierungszustand der LPS-behandelten Mikrogliazellen, sowie auf die Ausschüttung pro-inflammatorischer Zytokine und NO.

Mit der Möglichkeit der Impedanzmessung lässt sich der Aktivierungszustand von Mikrogliazellen nach Behandlung mit LPS sozusagen live verfolgen. Bisherige Untersuchungen an primären Neuronen zeigten, dass sich mit der Änderung der Morphologie und Viabilität der Zellen das Impedanzsignal dementsprechend verändert (Diemert et al. 2012). Auf Mikrogliazellen angewendet, lässt sich durch die morphologische Änderung bei Aktivierung der Zellen eine vergrößerte Impedanz feststellen und über einen bestimmten Zeitpunkt verfolgen (Dolga et al. 2012). Interessanterweise geschieht diese morphologische Änderung nach LPS-Stimulation schon innerhalb von zwei Stunden. Auf diese initiale Veränderung der Impedanz folgt eine kurze Phase, in der das Impedanzsignal nicht weiter anstieg. Danach konnten wir eine Phase abgrenzen in der die Impedanz immer weiter ansteigt und dementsprechend auch der Aktivierungsstatus der Mikrogliazellen (Dolga et al. 2012). Dieser Kinetik entgegengesetzt ist die Veränderung des Ca^{2+}-Haushalts nach Aktivierung der Mikrogliazellen mit LPS. Nach einem raschen Anstieg intrazellulärer Kalziumspiegel fällt dieser rasch wieder aufs Ausgangsniveau ab (Dolga et al. 2012). Diese Ergebnisse gehen mit denen von Beck *et al.* einher, die ein ganz ähnliches Aktivierungsprofil von Kalziumkanälen nach LPS-Behandlung gemessen haben (Beck et al. 2008). Diese Daten passen sehr gut zum kinetischen Profil des SK-Kanal-Aktivators CyPPA. Erst nach sieben bis acht Stunden konnten wir einen signifikanten Einfluss auf das Impedanzsignal und somit den Aktivierungsstatus der Mikrogliazellen nach Stimulation mit LPS messen (Dolga et al. 2012). Diese Änderung im Aktivierungsstatus schlägt sich auch auf die Ausschüttung pro-inflammatorischer Zytokine und NO nieder – sowohl zeit- als auch dosisabhängig ließ

sich eine Reduktion dieser Entzündungsparameter messen. Unsere Experimente zeigen, dass lediglich das pro-inflammatorische Zytokin IL-6 abhängig vom Kalziumspiegel ausgeschüttet wird, da IL-6-Level durch die Gabe des Ca^{2+}-Chelators EDTA nach LPS-Gabe gesenkt werden konnten. TNF-α-Level waren hiervon nicht beeinflusst (Dolga et al. 2012). Mit der gleichzeitigen Applikation von verschiedenen SK-Kanal inhibitorischen Peptiden konnte für die Ausschüttung von IL-6 und NO, nicht aber für TNF-α, eine Abhängigkeit des SK3-Kanals festgestellt werden (Dolga et al. 2012). Diese differentielle Zytokininduktion von IL-6 und TNF-α durch veränderte Kalziumlevel wurde auch schon in Myozyten gezeigt (Keller et al. 2006). In Myozyten scheint eine Steigerung der IL-6 Produktion durch erhöhte Kalziumlevel Calcineurin zu aktivieren, was wiederum den Transkriptionsfaktor NFAT (englisch: *nuclear factor of activated T-cells*) aktiviert. Die Ausschüttung von TNF-α ist in diesem Modellsystem unabhängig von intrazellulären Kalziumleveln und NFAT. Diese differentielle Kontrolle der Zytokinexpression und Zytokinausschüttung wirft weitere Fragen auf und die verschiedenen molekularen Mechanismen in der intrazellulären Signalweiterleitung hierfür müssen noch untersucht werden und sind Gegenstand weiterer Untersuchungen. Die Veränderung der intrazellulären Kalziumspiegel alleine kann jedoch nicht Auslöser der verringerten Zytokin- und NO-Ausschüttung sein, wie Hoffmann *et al.* in ihrer Arbeit zur Beeinflussung intrazellulärer Kalziumströme zur Zytokinausschüttung zeigen (Hoffmann et al. 2003). Die alleinige Behandlung mit dem Ionophor Ionomycin, das intrazelluläre Kalziumspiegel anhebt, hatte keinen Effekt auf die Zytokinausschüttung von mikroglialen Zellen. Ein weiterer Mechanismus muss durch die Aktivierung der SK-Kanäle beeinflusst werden. Denkbar wäre hier ein Einfluss auf Ryanodin-Rezeptoren und Inositoltrisphosphat-sensitiven Kalzium-Kanäle, da diese räumlich eng mit SK-Kanälen assoziiert sind, funktionell in Mikrogliazellen exprimiert sind und ein Zusammenhang in neuronalen Zellen gegeben ist (Klegeris et al. 2007; Dolga und Culmsee 2012).

SK-Kanäle bieten sich somit als spezifisches Ziel für immunmodulatorische Eingriffe im zentralen Nervensystem an. Zusätzlich wirkt die Aktivierung von SK3-Kanälen in Neuronen über negative Feedbackloops Exzitotoxizität entgegen (Kuiper et al. 2012) – ein schöner Nebeneffekt. Gerade bei AD scheint eine Dysregulation der Kalzium-Homöostase ein wichtiger Faktor für die Entstehung und das Fortschreiten der Erkrankung zu sein (LaFerla 2002). Bereits bevor klinische Symptome feststellbar sind, besteht eine Dysregulation der Kalzium-Homöostase in Fibroblasten, die aus Familienmitgliedern von Alzheimer-Patienten isoliert wurden die später an AD erkrankten (Etcheberrigaray et al. 1998).

3.3 Ausblick

Sowohl nAbs-Aβ als auch CyPPA haben, neben ihrer bisherigen bekannten protektiven Auswirkungen auf Neurone, Einfluss auf die Aktivierung mikroglialer Zellen. Da viele Neurodegenerative Erkrankungen mit einer Neuroinflammation einhergehen, sind beide Agenzien in der Theorie hervorragend als Alzheimer-Therapeutikum geeignet. Ausführliche Untersuchungen im Alzheimer-Tiermodell sind allerdings noch vonnöten, um sowohl die Wirksamkeit als auch die Verträglichkeit zu verifizieren.

4. Referenzen

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5. Zusammenfassung

Die Alzheimer-Krankheit (AD) ist die weltweit häufigste, nicht kausal behandelbare, neurodegenerative Erkrankung. Charakteristisch für AD ist der neuronale Zelluntergang, die Bildung von Amyloid-Beta (Aβ)-Plaques und Neurofibrillen sowie eine Aktivierung mikroglialer Zellen und damit einhergehende Neuroinflammation. Oligomere Formen des Aβ-Peptids scheinen dabei besonders toxisch für die Neurone und immunstimulatorisch für die Mikrogliazellen zu sein. Durch die Modulation der Alzheimer-assoziierten Neuroinflammation könnte der Verlauf von AD positiv beeinflusst werden. In der vorliegenden Arbeit wurde der Einfluss von natürlich vorkommenden Autoantikörpern gegen Aβ (nAbs-Aβ) und CyPPA (Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine), einem SK-Kanal-Aktivator, auf die Aktivierung primärer mikroglialer Zellen untersucht.

Um die Wirkung von nAbs-Aβ auf Aβ-Oligomer-behandelte Mikrogliazellen zu untersuchen, wurde zunächst der Einfluss von nAbs-Aβ auf die Viabilität Aβ-behandelter mikroglialer Zellen untersucht. nAbs-Aβ zeigten keinen Einfluss auf die durch Aβ-Oligomere hervorgerufene Reduktion in der Viabilität. Allerdings konnte ein Anstieg der Phosphorylierung der MAP-Kinase p38 mit einhergehendem Anstieg der pro-inflammatorischen Zytokine IL-6 und TNF-α bei Co-Administration von nAbs-Aβ mit Aβ-Oligomeren *in vitro* gemessen werden. Die Aufnahme von Aβ-Oligomeren aus dem Extrazellularraum in mikrogliale Zellen verdoppelte sich durch die Behandlung mit nAbs-Aβ. Interessanterweise hat der Einfluss von nAbs-Aβ auf Viabilität, Zytokinausschüttung und Phagozytose auf Aβ-behandelte Mikrogliazellen einen positiven Effekt auf primäre Neurone *in vitro*. Hierfür wurden primäre Neurone in Überständen von behandelten Mikrogliazellen kultiviert. Primäre Neurone, die in Überständen von Mikrogliazellen, die sowohl mit nAbs-Aβ als auch mit Aβ-Oligomeren behandelt wurden, kultiviert wurden, zeigten eine signifikant höhere Viabilität als solche, die in Mikroglia-Überständen kultiviert wurden, die nur mir Aβ-Oligomeren behandelt wurden. Der Einfluss von nAbs-Aβ auf die Alzheimer-assoziierte Neuroinflammation wurde auch *in vivo* in Tg2576 Mäusen untersucht. Dabei zeigte sich ein altersabhängiger Effekt: in jungen Tieren zeigte sich kein Effekt von nAbs-Aβ, wohingegen in alten Tieren die Sekretion sämtlicher pro-inflammatorischer Zytokine durch die einmalige Applikation von nAbs-Aβ auf Wildtyp-Niveau abgesenkt werden konnte.

Auch mit der Anwendung der zweiten Substanz, CyPPA, auf primäre mikrogliale Zellen konnte der Aktivierungszustand der Zellen moduliert werden. Dies wurde mit zwei unterschiedlichen Herangehensweisen untersucht. Einerseits konnte mit Impedanz-basierten Messungen gezeigt werden, dass sich die Morphologie LPS-aktivierter

Mikrogliazellen durch die Behandlung mit CyPPA der Morphologie unbehandelter Zellen angleicht. Zum anderen hatte CyPPA einen inhibierenden Einfluss auf die Sekretion pro-inflammatorischer Zytokine und des pro-inflammatorischen Signalmoleküls NO. Durch die Anwendung SK-Kanal Subtyp spezifischer inhibitorischer Peptide konnte für IL-6 und NO – nicht aber für TNF-α, eine Abhängigkeit der SK3-Kanal-Aktivität gezeigt werden. Zudem ist die Ausschüttung von IL-6 Ca^{+2}-abhängig.

Zusammenfassend konnte gezeigt werden, dass durch die Applikation von nAbs-Aβ *in vivo* keine Zunahme der Neuroinflammation zu beobachten ist. Bei sehr alten Tieren konnte sogar eine anti-inflammatorische Wirkung gezeigt werden. Zusätzlich lässt sich *in vitro* mit der Applikation von nAbs-Aβ eine deutliche Steigerung der Phagozytoserate von Aβ-Oligomeren feststellen. Der modulierende Effekt von nAbs-Aβ auf Mikrogliazellen wirkt sich zudem indirekt auf die neuronale Viabilität aus. Die Anwendung dieser körpereigenen Antikörper bietet im Vergleich zu monoklonalen Antikörpern oder aktiven Immunisierungsstrategien den Vorteil geringerer Nebenwirkungen, da dem Körper mit der Behandlung natürlich vorkommender Autoantikörper keine körperfremden Substanzen verabreicht werden und somit eine Aktivierung des Immunsystems unwahrscheinlich wird. Der Einsatz von CyPPA *in vitro* zeigte vielversprechende Wirkung auf aktivierte Mikrogliazellen und sollte Gegenstand weiterer *in vivo* Studien sein. Da sich beide Substanzen auch positiv auf Neurone auswirken, sind sie vielversprechende Kandidaten für die Modulation der Alzheimer-assoziierten Neuroinflammation.

6. Summary

Alzheimer's Disease (AD) is the most frequent neurodegenerative disorder. Besides the loss of cholinergic neurons, AD is characterized by plaque deposition, the formation of neurofibrillary tangles as well as microglial activation. The formation of toxic amyloid-β (Aβ) oligomers is crucial in the degenerative process and leads to synaptic dysfunction and neuronal apoptosis, but also neuroinflammation. Neuroinflammation is a possible starting point for a therapeutic strategy. Here we investigated the effect of naturally occurring autoantibodies against Aβ (nAbs-Aβ) and the SK-channel activator CyPPA (Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine) on microglial activation.

First, we investigated the effect of nAbs-Aβ on the viability of Aβ-oligomer treated microglial cells. There was no effect on the oligomer-induced reduction of cell viability, whereas there was an increase in the phosphorylation state of the stress pathway p38 MAP Kinase upon co-treatment with nAbs-Aβ and Aβ-oligomers. This rise in p38 phosphorylation was accompanied by an increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α. In addition, the uptake of Aβ-oligomers by microglial cells was enhanced following nAbs-Aβ-treatment. Interestingly, the impact of nAbs-Aβ on Aβ-treated microglial cells' viability, cytokine secretion and phagocytosis *in vitro* exerted beneficial effects on primary neurons. We administered supernatants of treated microglial cells to primary neurons and measured the neurons' viability. Primary neurons that were treated with supernatants of microglial cells co-treated with nAbs-Aβ and Aβ showed a significantly increased viability compared to neurons treated with supernatants of cells treated with Aβ alone. We also investigated the effect of a single dose of nAbs-Aβ in Tg2576 mice and evaluated the levels of pro-inflammatory cytokines in the brain. The cytokine inhibitory property of nAbs-Aβ *in vivo* seems to be age-dependent. In very old animals there was a reduction in cytokine-levels, whereas in younger animals there was no. With the application of CyPPA we were also able to modulate the activation state of microglial cells. Morphological changes, measured with real time impedance, as well as NO- and cytokine-production induced with LPS could be prevented with the application of CyPPA. Using specific inhibitory peptides for the SK-channel subtypes, we were able to show a SK3-channel dependency for the secretion of IL-6 and NO, but not for TNF-α. IL-6 secretion was also dependent on extracellular calcium, leading to the hypothesis that CyPPA reduces the activation of microglial cells via modulating calcium homeostasis.

In summary we were able to provide insight in the mechanism of action of nAbs-Aβ on microglial cells *in vitro*. In addition the effects of nAbs-Aβ on microglial cells could be

conveyed to primary neurons. Interestingly our *in vivo* data indicate that nAbs-Aβ should be considered for therapeutic use as there is no induction of neuroinflammation. The second substance, CyPPA, should be investigated further concerning its immune-modulating ability. Both data are very promising and point to a new way of modulating microglial activation. As both substances are also known to exert beneficial effects on neurons, these two approaches are promising candidates of modulating Alzheimer-associated neuroinflammation.

7. Anhang

Abkürzungsverzeichnis

Abb	Abbildung
Aβ	Amyloid-β
AD	Englisch: *Alzheimer's disease*, Alzheimer-Krankheit
AICD	Englisch: *Amyloid-Precursor-Protein intracellular domain*
APP	Englisch: *Amyloid-Precursor-Protein*, Amyloid-Vorläuferprotein
BDNF	Englisch: *Brain-derived neurotrophic factor*
CyPPA	Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amin
CSF	Englisch: *Cerebrospinal fluid*, Zerebrospinalflüssigkeit
EDTA	Ethylendiamintetraacetat
ELISA	Englisch: *Enzyme-linked immunosorbent Assay*
FITC	Fluoresceinisothiocyanat
ft	Englisch: *Flow-through*
GDNF	Englisch: *Glia-derived neurotrophic factor*
IgG	Immunglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IVIg	Intravenöse Immunglobuline
LPS	Lipopolysaccharid
MAP	Englisch: *Mitogen-activated protein*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
nAbs	Englisch: *Naturally occurring autoantibodies*, natürlich vorkommende Autoantikörper
NFAT	Englisch: *nuclear factor of activated T-cells*
NGF	Englisch: *Nerve growth factor,* Nervenwachstumsfaktor
NO	Stickstoffmonoxid
NT	Neurotrophin
sAPPα	Lösliches Amyloid-Precursor-Protein Spaltprodukt der α-Sekretase
sAPPβ	Lösliches Amyloid-Precursor-Protein Spaltprodukt der β-Sekretase
SK	Englisch: *Small conductance*
TIP60	Englisch: *Tat-interactive protein 60*
TNF-α	Tumornekrosefaktor-α

Abbildungsverzeichnis

Abb. 1: Bildung von Aβ aus dem Amyloid-Vorläuferprotein 2

Verzeichnis der akademischen Lehrer

Meine Akademischen Lehrer waren die Damen und Herren der Philipps-Universität Marburg:

Aigner, Bauer S., Bauer U., Becker, Bette, Boekhoff, Brehm, Bröring, Büch, Burchert, Czubayko, Daut, Del Rey, Eilers, Elsässer, Fritz, Feuser, Garten, Glorius, Grzelinski, Gudermann, Hasilik, Hassel, Huber, Homberg, Jacob, Kira, Kirchner, Knöller, Koch, Lill, Liss, Löffler, Lohoff, Lüers, Maisner, Matrosovich, Moll, Mühlberger, Müller, Müller-Brüsselbach, Renigunta V., Roeper, Röhm, Schäfer, Schütz, Suske, Weihe, Westermann, Voigt.

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Publikationen

1. **Gold, M.**, D. Mengel, S. Roskam, R. Dodel und J. P. Bach (2013). Mechanisms of action of naturally occurring antibodies against beta-amyloid on microglia. J Neuroinflammation. 10: 5 10.1186/1742-2094-10-5.

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Weitere Publikationen

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Kongressbeiträge

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Gold, M. *In-vitro* mechanisms of action of naturally occurring autoantibodies against Aβ. Vortrag, 8th International congress on autoimmunity (Mai 2012) Granada, Spanien.

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Mechanisms of action of naturally occurring antibodies against β-amyloid on microglia

Maike Gold, David Mengel, Stephan Röskam, Richard Dodel* and Jan-Philipp Bach

Abstract

Background: Naturally occurring autoantibodies against amyloid-β (nAbs-Aβ) have been shown to exert beneficial effects on transgenic Alzheimer's disease (AD) animals *in vivo* and on primary neurons *in vitro*. Not much is known about their effect on microglial cells. Our aim was to investigate the effect of nAbs-Aβ on amyloid-β (Aβ)-treated microglial cells *in vitro* with respect to cell viability, stress pathways, cytokine production and phagocytotic abilities and whether these effects can be conveyed to neurons.

Methods: Primary microglial cells isolated from Swiss Webster mouse mesencephalons on embryonic day 13.5 were pretreated with nAbs-Aβ and then treated with Aβ oligomers. After 3 hours, phagocytosis as well as western blot analysis were evaluated to measure the amount of phagocytized Aβ. Cell viability was analyzed using an MTT assay 24 hours after treatment. Pro-inflammatory cytokines in the supernatants were analyzed with ELISAs and then we treated primary neuronal cells with these conditioned microglia supernatants. Twenty-four hours later we did a MTT assay of the treated neurons. We further investigated the effect of a single nAbs-Aβ administration on Tg2576 mice *in vivo*.

Results: Upon co-administration of Aβ and nAbs-Aβ no change in microglia viability was observed. However, there was an increase in phosphorylated p38 protein level, an increase in the pro-inflammatory cytokines TNF-α and IL-6 and an increase in Aβ uptake by microglial cells. Treatment of primary neurons with conditioned microglia medium led to a 10% improvement in cell viability when nAbs-Aβ were co-administered compared to Aβ-treated cells alone. We were unable to detect changes in cytokine production in brain lysates of Tg2576 mice.

Conclusions: We provide evidence on the mechanism of action of nAbs-Aβ on microglia *in vitro*. Interestingly, our *in vivo* data indicate that nAbs-Aβ administration should be considered as a therapeutic strategy in AD, since there is no inflammatory reaction.

Keywords: Alzheimer's disease, β-amyloid, Microglia, Inflammation, Immunoglobulins

Background

Alzheimer's disease (AD) is a neurodegenerative disorder leading to the loss of cholinergic neurons in the brain, characterized by extracellular amyloid-β (Aβ) deposits and the formation of neurofibrillar tangles comprising hyperphosphorylated tau protein inside the cell. The toxicity of Aβ deposits is conveyed by oligomeric aggregates that lead to synaptic dysfunction and apoptosis of neurons [1,2].

Active immunization strategies using different forms of Aβ have been extensively tested in amyloid precursor protein (APP) transgenic mouse models. The initial animal results were promising [3,4], but since active immunization in AD patients was associated with considerable side effects [5] and some patients did not develop Aβ-antibody titers [6], passive immunization strategies came into focus. Treatment with humanized monoclonal antibodies [7] as well as passive immunization with intravenous immunoglobulins (IVIg) has been investigated in pilot clinical trials [8,9]. The rationale for its use is that IVIg contain naturally occurring autoantibodies against Aβ (nAbs-Aβ). Naturally occurring autoantibodies in the central nervous system are involved in maintaining homeostasis by removing debris and are known to prevent inflammation [10]. The beneficial effects of nAbs-Aβ have been shown both *in vitro* on primary neurons and neuronal cell lines as well as *in vivo*

* Correspondence: dodel@med.uni-marburg.de
Department of Neurology, Philipps-University of Marburg, Baldingerstraße 1, Marburg 35043, Germany

in transgenic mice [11,12]. The effect of IVIg on microglial cells has already been investigated by other groups. It has been shown that IVIg reduce phagocytosis *in vitro* via Fc receptors [13], IVIg induce tumor necrosis factor-α (TNF-α) and nitric oxide (NO) in a dose-dependent manner, whereas the greater the IgM/IgA content the higher the impact on microglial cells [14], and that IVIg enhance the secretion of matrix metalloproteinase 9, which seems to play a role in the pathogenesis of multiple sclerosis [15]. Referring to Aβ, Magga *et al*. found an increase in Aβ clearance when they administered IVIg in a dose-dependent manner on primary microglial cells [16]. IVIg depleted of nAbs-Aβ did not promote clearance, indicating that the enhanced clearance effect is mediated by nAbs-Aβ. In a study on the immortalized murine microglial cell line BV-2, administration of IVIg restored cells' viability and enhanced phagocytotic ability of fibrillar Aβ [17]. In addition to these results, IVIg are limited in their availability and we therefore decided to use nAbs-Aβ in our study. Theoretically, these will be able to be cloned eventually and therefore the problem of availability can be overcome. In all experiments we used the negative fraction of IVIg, which contains all antibodies except those that were purified with the affinity chromatography, and we were not able to see the same effects as those obtained with nAbs-Aβ, coming to the conclusion that nAbs-Aβ are the active substance in IVIg responsible for the beneficial effects with regard to AD. The effect and the interaction, however, of nAbs-Aβ on microglial cells have not been investigated to date. This is especially important as microglia are purported to have a major role in the pathogenesis and propagation of AD [18]. Upon stimulation, microglial cells can react in many different ways, including phagocytosis, the secretion of immunomodulating cytokines and finally programmed cell death in order to kill pathogens or restore tissue integrity [19]. Activated microglial cells have been found surrounding plaques in histopathological sections of AD patients [20] and their role in the phagocytosis of all forms of Aβ has been investigated. Due to the immune-stimulating effect of foreign monoclonal antibodies in the human body, we were interested in the effect of nAbs-Aβ on primary microglial cells. In the following experiments we investigated the effect of nAbs-Aβ on Aβ-treated microglial cells with respect to cell viability, neuroinflammation and phagocytosis, and whether any effect on primary neurons is conveyed by treated microglial cells.

Material and methods

All chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA unless indicated otherwise.

Antibodies

Antibodies against the following proteins were used: Aβ (clone 6E10, Covance, Princeton, NJ, USA), phosphorylated (phospho) p38, horseradish peroxidase (HRP)-conjugated secondary antibodies (all Cell Signaling Technology, Danvers, MA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Novus Biologicals, Littleton, CO, USA), allophycocyanin (APC)-conjugated CD11b (eBioscience, San Diego, CA, USA).

Cells

Primary microglial cell culture

For the isolation of primary microglial cells we used a protocol based on that described by Saura *et al*. [21]. Microglial cells were derived from embryonic day 13.5 (E13.5) Swiss Webster mouse mesencephalons. Briefly, meninges-free mesencephalons were isolated, collected and homogenized in Leibovitz L-15 medium (PAA Laboratories, Pasching, Austria). After centrifugation cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), 100 U/mL penicillin and 100 μg/mL streptomycin (Lonza, Basel, Switzerland) and plated on polyethyleneimine (PEI)-coated plates. After 7 days cells were stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Roche, Basel, Switzerland). Cells were subplated on PEI-coated plates and used for experiments on day *in vitro* 14 to 19.

Primary cortical neuron cell culture

Neurons were cultured from cortices of E13.5 Swiss Webster mice. Briefly, meninges-free cortices were isolated, collected and homogenized in Leibovitz L-15 medium and resuspended in Neurobasal-A Medium (Invitrogen, Grand Island, NY, USA) supplemented with B27 (Gibco, Basel, Switzerland), 100 U/mL penicillin and 100 μg/mL streptomycin and L-glutamine and plated on PEI-coated plates. Cells were used for experiments on day 6 to 8.

Animals

Twenty- to 22-month-old heterozygous adult female Tg2576 mice expressing mutant $APP_{SWE\ (695(K670N,M671L)}$ under the control of the hamster prion promoter in a hybrid C57Bl/6 × SJL background and age- and gender-matched non-transgenic wild-type control mice (WT) were used for all experiments. Tg2576 and WT mice were randomly divided into groups of five to six, independent of genotype and treatment, on a 12 hour light–dark schedule (lights on 07:00 to 19:00). They had free access to tap water, were fed *ad libitum* and kept under standard conditions. The sample sizes of the groups were as follows: transgenic (Tg) control n = 5, Tg nAbs-Aβ n = 5, WT n = 6. All animal procedures were approved by the office of the district president and the Institutional Animal Care and Use Committee.

Administration of nAbs-Aβ to mice

Mice were treated intraperitoneally (i.p.) with nAbs-Aβ (400 μg dissolved in 0.2 ml of physiological saline solution) or vehicle (0.2 ml physiological saline solution). The mice were sacrificed 24 hours later and samples were taken. Brains were immediately frozen in liquid nitrogen and stored at −80°C.

Preparation of oligomeric Aβ

Aβ oligomers were synthesized according to Kayed *et al*. [22]. Briefly, lyophilized synthetic $A\beta_{42}$ peptide (PSL, Heidelberg, Germany) was initially monomerized by dissolving in hexafluoroisopropanol (HFIP) and separated into aliquots in low-binding tubes. HFIP was evaporated and aliquots were stored at −20°C until use. The peptide film was resuspended in ultrapure water with a final concentration of 232 μM. A small magnetic stirrer was added and the peptide solution was stirred for 48 hours at room temperature at 1400 rpm.

Preparation of nAbs-Aβ

nAbs-Aβ were isolated from IVIg as previously described [11]. Briefly, we used purified intravenous IgG (Octagam 5%). Ninety-six percent of protein represents normal human IgG (IgA <0.2 mg; IgM <0.1 mg). IgG subclasses are fully represented (IgG 1, 65%; IgG 2, 30%; IgG 3, 3%; IgG 4, 2%). To ensure homogenous orientation of the Aβ peptide to the affinity column, a cysteine residue was introduced at the N-terminal part of the peptide. The azlactone-activated support contains an iodoacetyl group (Ultralink; Perbio, Bonn, Germany) at the end of a hexadecyl-spacer group, which reacted with the cysteinyl-sulfhydryl group to yield a stable thioether linkage in order to reduce steric hindrance and provide maximum binding capacity of the antibodies. The bound antibodies were eluted from the column with 10 × 0.5 ml 0.1 M glycine buffer, pH 2.8. Each fraction was collected in a microreaction tube containing 35 μl 1 M Tris–HCl, pH 9. IVIg depleted of nAbs, termed flow-through (ft), was also collected and used as a negative control in experimental settings. To maintain the integrity of the antibodies, a neutral pH was adjusted immediately after elution by adding the appropriate amount of Tris-HCI or glycine buffer. nAbs-Aβ and ft were sterile filtered and stored at −20°C until use.

Cell viability assessment

Quantification of cell viability was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Microglial cells were treated with oligomeric $A\beta_{42}$ (5 μM) and/or nAbs-Aβ or ft (both 0.1 μM and 1 μM) for 24 hours in DMEM without FBS. Medium was changed to medium containing 0.5 mg/mL MTT and incubated for 1 hour. Medium was removed and cells were solubilized with dimethyl sulfoxide (DMSO) (AppliChem, Darmstadt, Germany) and shaken for 30 minutes. The absorbance was measured at 570 nm on a plate reader. Neuronal cells were treated with supernatants of microglial cells. Neurobasal-A medium was removed and conditioned medium from microglial cells was added. After 24 hours, medium was replaced with DMEM containing 0.5 mg/mL MTT and incubated for 1 hour and the assay was performed as described above.

Measurement of TNF-α, IL-1β, IL-6 and interferon-γ

Primary microglial cells were pre-treated with nAbs-Aβ or ft (0.1 μM) for 30 minutes and stimulated with oligomeric $A\beta_{42}$ (5 μM) for 24 hours. Supernatants were stored at −20°C until use. Mice brains were lysed using T-PER lysis buffer (Pierce, Rockford, IL, USA) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using a spectrophotometer. Concentrations of TNF-α, interleukin-1β (IL-1β), IL-6 and interferon-γ (IFN-γ) were quantified using the Duoset enzyme-linked immunosorbent assay (ELISA) system (R&D, Minneapolis, MN, USA) according to the manufacturer's protocol.

Western blot

Treated cells were washed twice with ice-cold phosphate-buffered saline (PBS) and cells were lysed using M-Per supplemented with protease and phosphatase inhibitors according to the manufacturer's protocol. The protein concentration of the cell lysates was determined using NanoDrop. Total cell protein was separated by 4 to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked with 1 × Rotiblock (Carl Roth, Karlsruhe, Germany) and incubated with the appropriate primary antibody dilutions for Aβ (1:2000), phospho p38 (1:4000) or GAPDH (1:5000). Membranes were washed with Tris-buffered saline containing 5% Tween20 and incubated with secondary antibodies. Membranes were incubated with SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA) and exposed to an autoradiographic film (CL-Xposure Film, Thermo Scientific, Rockford, IL, USA). Band intensity was quantified using a densitometer.

Aβ phagocytosis assay

Fluorescein isothiocyanate (FITC)-labeled $A\beta_{42}$ was oligomerized as described above. Primary microglial cells were pre-incubated with nAbs-Aβ or ft (0.1 μM) in serum-free DMEM for 30 minutes at 37°C and then treated with FITC-$A\beta_{42}$ (5 μM) for 3 hours. For flow cytometric analysis, microglial cells were rinsed twice with ice-cold PBS, harvested, and washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 0.1% FBS). Microglial cells were stained with APC-

conjugated CD11b antibody, washed and resuspended in FACS buffer containing HOECHST 33258 to sort vital cells only. Measurements were performed with a LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Only vital and $CD11b^+$ cells were used for uptake comparison. Uptake of the differently treated cells was evaluated by comparing the mean fluorescence intensity of FITC. Levels of Aβ phagocytosis were also determined by Western blot.

Statistical analysis

All results of *in vitro* as well as *in vivo* experiments are presented as the mean ± SD. We used the Student's *t* test to assess the statistical significance of all experiments. For all statistical comparisons, the following definitions were used: $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

Results

nAbs-Aβ are not toxic to microglia

nAbs-Aβ have been shown to have beneficial effects on Aβ oligomer-induced toxicity in neuronal cells [11,12]. It has recently been reported that Aβ oligomers reduce viable microglial cells *in vitro* [23]. We investigated the effect of nAbs-Aβ on Aβ oligomer-induced toxicity (Figure 1). Cell viability was reduced to 50% when stimulated with $Aβ_{42}$ oligomers. nAbs-Aβ had no beneficial effect on the Aβ-induced reduction in viability. nAbs-Aβ alone showed no reduction in cell viability, nor did solvent-treated cells.

Effect of Aβ oligomers and co-administered nAbs-Aβ on stress pathways and cytokine levels *in vitro*

Since Aβ-mediated cell viability was unaffected by nAbs-Aβ, we investigated changes in intracellular stress pathways upon stimulation with Aβ oligomers and in the presence of nAbs-Aβ. We observed an increase in phospho p38 protein levels upon stimulation with Aβ oligomers alone compared to untreated microglial cells (Figure 2A). nAbs-Aβ alone also induced a slight increase in phospho p38 levels. However, co-administration of Aβ oligomers and nAbs-Aβ led to a strong induction of phospho p38 with protein levels increasing 10-fold compared to untreated cells (Figure 2B).

Cytokine levels were examined after 24 hours co-treatment. The results are consistent with the results obtained from the phospho p38 stress-pathway investigation: Aβ and nAbs-Aβ alone slightly increased TNF-α (Figure 2C) and IL-6 (Figure 2D) levels, whereas co-administration of both Aβ and nAbs-Aβ led to a strong and significant induction in pro-inflammatory cytokine levels. Co-administration of ft did not lead to the induction of cytokine levels (data not shown).

Influence of nAbs-Aβ on pro-inflammatory cytokines in Tg2576 transgenic animals

After observing the induction of pro-inflammatory cytokines *in vitro* following treatment of microglial cells with nAbs-Aβ, we next evaluated cytokine concentration in brain homogenates of wild-type animals as well as Tg2576 mice. For all analyzed cytokines we were able to detect upregulation in transgenic animals (Figure 3). IL-1β was elevated by 58% compared to wild-type animals (Figure 3A) (7.11 ± 2.05 vs. 11.23 ± 3.32, $P < 0.05$), IFN-γ levels (Figure 3B) in Tg2576 mice brain were increased by 41% (19.78 ± 1.48 vs. 27.91 ± 8.00, $P < 0.05$), TNF-α (Figure 3C) was increased by 24% (93.16 ± 7.97 vs. 115.62 ± 9.95, $P < 0.05$) and IL-6 (Figure 3D) showed an increase of 24% (19.65 ± 1.48 vs. 24.45 ± 4.32, $P < 0.05$), respectively. Treatment of Tg2576 mice with nAbs-Aβ did not lead to a significant induction or attenuation in

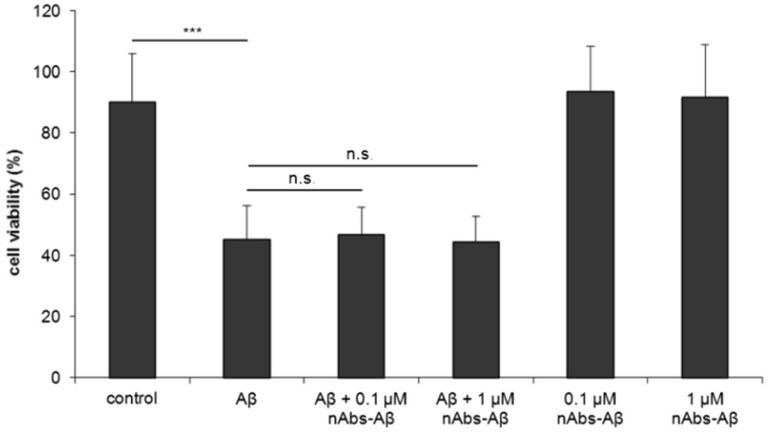

Figure 1 Aβ oligomers reduce cell viability, co-administered nAbs-Aβ do not attenuate cell death. Primary microglia cells were treated with $Aβ_{42}$ oligomers, cell viability was measured using the MTT assay. To investigate the effect of nAbs-Aβ, microglial cells were pre-incubated for 30 minutes with different concentrations of nAbs-Aβ. Viability of untreated cells was referred to as 100% viability. Experiments were performed at least three times, independently. nAbs-Aβ, naturally occurring autoantibodies against amyloid-β; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

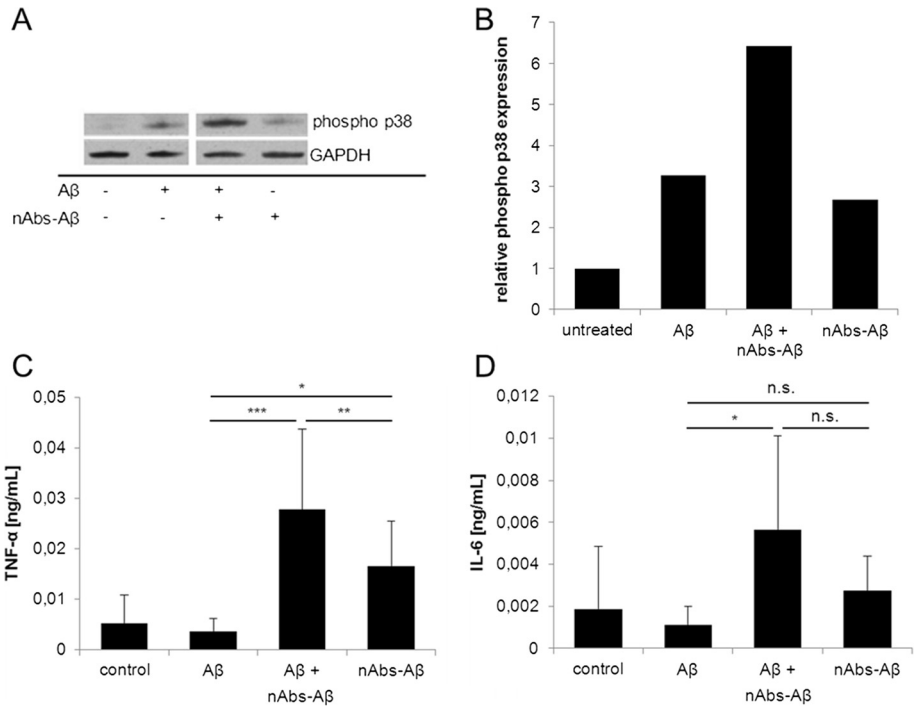

Figure 2 Co-administration of nAbs-Aβ and Aβ oligomers leads to a rise in phosphorylated p38 and TNF-α levels. Western blot analysis was performed to detect protein levels of phosphorylated p38. GAPDH was used as loading control **(A)**. Phospho p38 levels were evaluated densitometrically and normalized to GAPDH **(B)**. **C** + **D** show cytokine levels of Aβ-treated cells. Cells were treated for 24 hours and supernatants were subjected to cytokine ELISA. ELISA experiments were performed at least three times, independently. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; nAbs-Aβ, naturally occurring autoantibodies against amyloid-β; TNF-α, tumor necrosis factor-α.

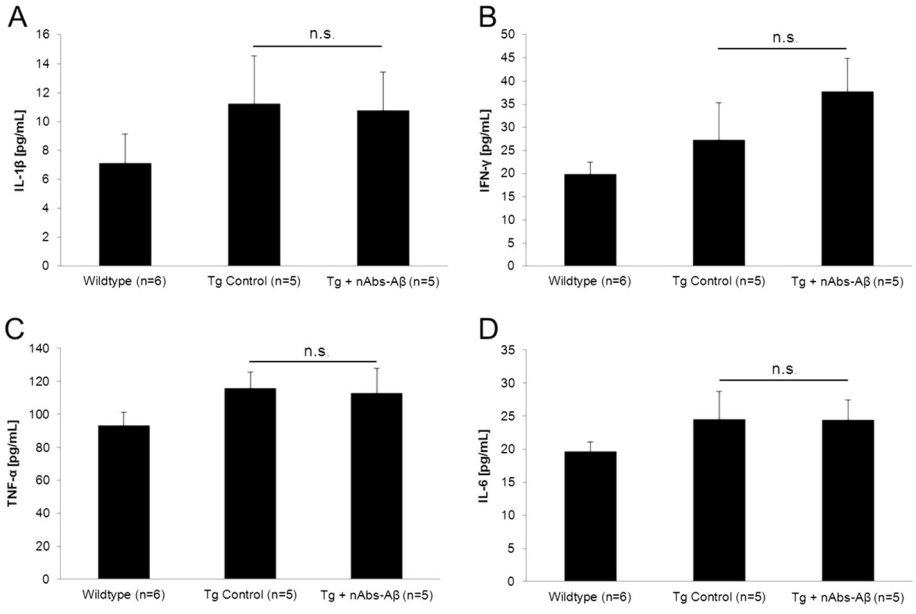

Figure 3 Injection of nAbs-Aβ does not change cytokine levels in Tg2576 mice brain lysates. Brain lysates of wild-type mice, Tg2576 mice and Tg2576 mice treated with nAbs-Aβ for 24 hours were subjected to cytokine ELISA. Figure 3 shows cytokine levels for IL-1β **(A)**, INF-γ **(B)**, TNF-α **(C)** and IL-6 **(D)**. ELISA, enzyme-linked immunosorbent assay; IL, interleukin; INF, interferon; nAbs-Aβ, naturally occurring autoantibodies against amyloid-β; TNF-α, tumor necrosis factor-α.

the brain concentration of any of the aforementioned cytokines.

nAbs-Aβ increase $A\beta_{42}$ uptake in primary microglial cells

Aβ is taken up by microglial cells from the periphery and it has been shown that the monoclonal antibody 6E10 increases the phagocytotic ability of microglial cells [24]. To investigate the effect of co-administration of Aβ oligomers and nAbs-Aβ on the phagocytotic abilities of microglial cells *in vitro*, we used FITC-labeled $A\beta_{42}$ and FACS analysis. Figure 4A shows a histogram of the shift in the mean fluorescence intensity of the cell population with nAbs-Aβ pre-treatment. Co-administration of nAbs-Aβ led to a doubling in the phagocytosis of $A\beta_{42}$ oligomers (Figure 4B). ft did not enhance phagocytosis, pointing to a genuine effect of nAbs-Aβ.

Levels of phagocytosed Aβ were also determined by western blotting (Figure 4C). The intensity of the protein bands was evaluated densitometrically (Figure 4D). As shown in Figure 4C and D the incubation of primary microglial cells with Aβ and nAbs-Aβ led to a doubling in phagocytosis. In contrast, no significant increase was observed when using ft.

Supernatants of nAbs-Aβ-co-treated microglial cells have beneficial effects on the viability of neurons compared to supernatants of Aβ-treated microglial cells

To investigate a possible indirect effect of nAbs-Aβ on primary neuronal cells, we cultured neurons in the medium of microglial cells treated with Aβ and/or nAbs-Aβ or ft for 24 hours and assessed cell viability using the MTT assay (Figure 5). Supernatants of microglial cells from three independent preparations were applied to neurons from three independent preparations. Compared to supernatants from solvent-treated microglial cells, supernatants from Aβ-treated microglial cells reduced the viability of neuronal cells to 67%. Following the co-administration of nAbs-Aβ to microglial cells, supernatants significantly reduced the number of viable neuronal cells to 77%. Pre-treatment with ft was not able to significantly restore the viability of primary neuronal cells. In addition, treatment with nAbs-Aβ alone was not harmful to neuronal cells.

Discussion

The positive effect of nAbs-Aβ on neuronal cells and the fact that IVIg may exert beneficial effects on cognition in AD patients has previously been established [9]. In addition AD patients have reduced levels of nAbs-Aβ in

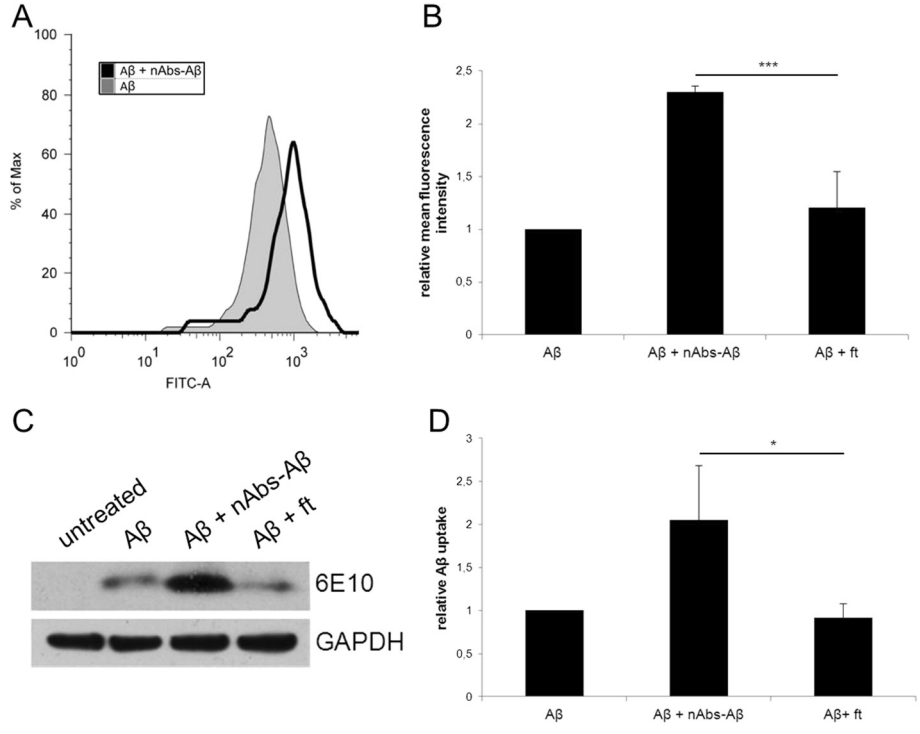

Figure 4 nAbs-Aβ enhance phagocytosis of Aβ oligomers in primary microglia. Histogram of the mean fluorescence intensity of FITC-Aβ and FITC-Aβ and nAbs-Aβ (0.1 μM) co-treated cells **(A)**. **(B)** shows the means of three independent experiments. Values were normalized to FITC-$A\beta_{42}$-treated cells. Cell lysates of $A\beta_{42}$-treated cells were also subjected to western blot. To detect Aβ uptake, blots were probed with the monoclonal Aβ antibody 6E10. GAPDH was used as loading control **(C)**. **(D)** shows the densitometric evaluation of three independent experiments; values are given normalized to GAPDH intensity. FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; nAbs-Aβ, naturally occurring autoantibodies against amyloid-β.

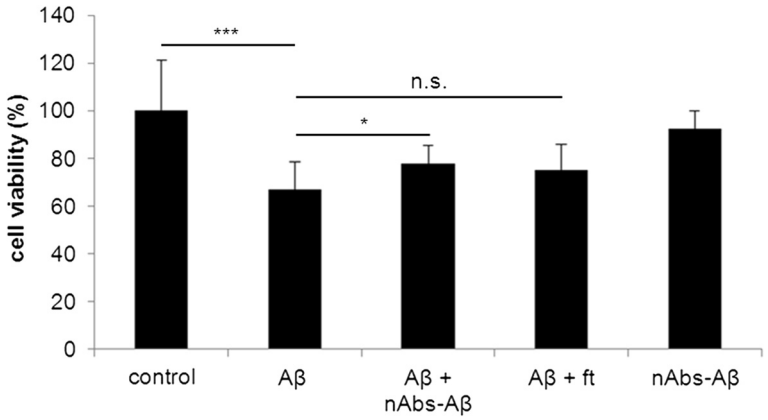

Figure 5 Beneficial effects of supernatants of nAbs-Aβ co-treated microglial cells on the viability of primary neurons. Supernatants of treated microglial cells were subjected to primary neurons and incubated for 24 hours. Aβ concentration was 5 μM and nAbs/ft concentration was 0.1 μM. Viability was assessed using MTT assay. Viability of control-treated cells was referred to as 100% viability. Figure 5 shows the means of at least three independent experiments. nAbs-Aβ, naturally occurring autoantibodies against amyloid-β; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

the CSF and plasma compared to age-matched controls, leading to the hypothesis that AD patients will have increased Aβ plaque deposits [25]. In this study we investigated the effect of nAbs-Aβ, isolated from IVIg, on Aβ-exposed microglial cells with the emphasis on cytokine production and phagocytosis of Aβ and whether changes in these physiological actions can convey beneficial effects to primary neurons.

Although the viability of neurons improves with the co-administration of nAbs-Aβ in Aβ oligomer-treated cells [11,12], we found no similar effects on the viability of microglial cells. nAbs-Aβ alone did not influence cell viability, an important property of nAbs-Aβ considering their therapeutic use. Smith *et al*. provided evidence for a link between increased phagocytosis of Aβ and induction of apoptosis in BV-2 microglial cells [26]. This may also hold true for the co-administration of nAbs-Aβ and could explain the difference in the effect observed for neuronal cells. Compared to the monoclonal antibody 6E10, nAbs-Aβ increased pro-inflammatory TNF-α levels *in vitro*, whereas no significant changes were observed for IL-6. With the additional administration of Aβ oligomers TNF-α levels and IL-6 levels rose significantly. Consistent with these results, we observed a strong increase in the phosphorylation of the stress pathway protein MAPK p38. There is contradictory evidence regarding whether pro-inflammatory cytokines are beneficial or harmful in AD; current data support the hypothesis that it is a matter of the right equilibrium [27]. Small rises in pro-inflammatory cytokines seem to have beneficial effects, whereas the induction of excessive and prolonged secretion of pro-inflammatory cytokines can lead to chronic neuroinflammation and neurodegeneration. Neuroprotective effects, especially of TNF-α, have been reported. The neuroprotective effect seems to be mediated by the regulation of peroxide formation, calcium accumulation and NF-κB activation [28]. It has been shown by several groups that Aβ overexpression induces neuroinflammation in the AD transgenic mouse models Tg2576 and B6.Cg-Tg (APPswe, PSEN1dE9)85Dbo/J [29,30]. In our present study, we also observed increased cytokine concentrations in 22-month-old Tg2576 mice compared to their wild-type littermates. In contrast to our *in vitro* experiments, administration of nAbs-Aβ to Tg2576 mice did not lead to an increased inflammatory reaction as measured by intracerebral cytokine levels. Instead IL-1β, TNF-α, IL-6 and IFN-γ levels were unchanged following the administration of nAbs-Aβ to Tg2576 mice. Although the brain is protected by the blood–brain barrier, it has already been shown that nAbs-Aβ can cross it [31]. *In vivo* studies provide an environment with astrocytes, neurons and microglial cells whereas our *in vitro* experiments were performed using microglial cells only. Von Bernhardi *et al*. observed that microglia exposed to Aβ respond with reactive morphological changes, induction of inducible nitric oxide synthase (iNOS), elevated nitric oxide production and decreased reductive metabolism. All these responses were attenuated by the presence of astrocytes [32]. Astrocytes are also involved in Aβ degradation and removal and play an important role in maintaining physiological homeostasis in the brain. However, compared to microglial cells they do not need a stimulus for phagocytosis and at the same time phagocytosis cannot be enhanced by large amounts of IVIg as it can be for microglial cells [16]. Further studies with mixed glial cell cultures are needed to investigate this specific issue.

Interestingly, our observations contrast with those of Minami and colleagues, who showed a profound induction of neuroinflammation following the administration

of monoclonal antibodies to mice [33]. One explanation might be the use of a different animal model in the aforementioned study compared to the Tg2576 mouse model in our study. More likely, as a passive immunization strategy, nAbs-Aβ may preclude a neuroinflammatory reaction, unlike monoclonal antibodies *in vivo*. As we only treated mice for 24 hours before measuring intracerebral cytokine levels one could argue that longer treatment periods are needed to assess the influence of administering anti-Aβ antibodies on neuroinflammation. A study by Puli *et al.* also showed significant suppression of TNF-α levels after long-term treatment with IVIg in the APP/PS1 mouse model of AD [34]. Nevertheless, long-term studies with repeated administration of nAbs-Aβ and ft are needed to investigate this issue further and to preclude a rise in pro-inflammatory cytokines upon chronic nAbs-Aβ administration in transgenic mice. With respect to the adverse effects of active immunization observed in an early clinical study, treatment with IVIg and/or nAbs-Aβ might prove a safer approach [5]. By applying two different methods we were able to show that phagocytosis of Aβ oligomers by microglial cells doubles when nAbs-Aβ are pre-administered. This effect was not observed with the same amount of flow-through, indicating a highly specific effect of nAbs-Aβ on the uptake of Aβ from microglial cells. This might be attributed to the formation of antigen-antibody complexes between nAbs-Aβ and Aβ oligomers, which are preferentially taken up by microglial cells. Phagocytosis of antigen-antibody complexes is usually mediated via the Fcγ receptor [35]. In the experimental setup that we used, microglial cells of murine origin were exposed to human IgG. Human Fcγ receptor 1 and murine Fcγ receptor 1 share 65 to 75% homology in their extracellular domains and it is known that human Fcγ receptor 1 can bind murine IgG [36]. Unfortunately, nothing is known on the ability of murine Fcγ receptor 1 to bind to human IgG. As our nAbs-Aβ preparation contains all IgG subclasses, it might be worth testing them with regard to their influence on cytokine secretion and phagocytosis separately in the future. Very recently Smith *et al.* developed a mouse model where all murine Fcγ receptors have been replaced by human Fcγ receptors [37]. It would be ideal to test our human nAbs-Aβ on this mouse model to verify our results and to exclude the impact of species on the immunoglobulin-Fc receptor interaction. However, the positive effect of nAbs-Aβ on Aβ phagocytosis is consistent with results obtained for monoclonal antibodies [24]. Since the amyloid hypothesis postulates that an increase in amyloid-β leads to neurodegeneration, nAbs-Aβ support Aβ oligomer clearance. To determine whether the changes observed for microglial cells have beneficial effects on neurons, we treated primary neurons with conditioned media from microglial cell cultures. Interestingly, we were able to observe a significant improvement in neuron viability from Aβ-treated microglial cells compared to those with co-administered nAbs-Aβ. This was not the case following co-administration of flow-through. We can exclude that this effect is due to a lower amount of Aβ in nAbs-Aβ-treated cell supernatants, even though we observed an increase in Aβ uptake. It is possible that in the co-treated supernatants Aβ is bound to nAbs-Aβ or even modulated in its aggregation state and is therefore less toxic to the neuronal cells. Another explanation could be that the activated microglial cells secrete neurotrophic factors, as it has been reported for lipopolysaccharide (LPS)-treated microglial cells by Nakajima *et al.* [38]. It is thus likely that simultaneous stimulation of Aβ and nAbs-Aβ activates the same signaling pathways in microglial cells that lead to the production of neurotrophic factors. We measured one neurotrophic factor (brain-derived neurotrophic factor (BDNF); data not shown) but were unable to show a significant increase. Other factors such as glial cell line-derived neurotrophic factor (GDNF) may play a role in the observed effect, and further studies are currently underway.

Conclusions

In summary, we investigated the effects of nAbs-Aβ isolated from IVIg on microglial cells. Although we were not able to rescue microglial cells upon stimulation with Aβ oligomers we showed that nAbs-Aβ have positive effects on the phagocytotic ability of Aβ oligomers. The beneficial effect of microglial-conditioned media on primary neurons is promising, and should be the focus of further research. Our present data demonstrate that nAbs-Aβ are able to enhance Aβ degradation and support neuronal survival without inducing a cerebral inflammatory reaction as shown by unchanged pro-inflammatory cytokine levels *in vivo*. These findings support nAbs-Aβ as a potential therapy to ameliorate Aβ-induced neuronal toxicity in AD.

Competing interests

This work was partially financed by the Alzheimer Forschungsinitiative (AFI) awarded to JPB.

Authors' contributions

MG and DM carried out the laboratory experiments. MG, DM and JPB analyzed the data, interpreted the results and wrote the paper. SR designed and carried out the animal experiments. RD discussed analyses, interpretation and presentation. In addition, he participated in the drafting of the paper. All authors have contributed to, read and approved the final manuscript.

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Naturally occurring autoantibodies interfere with β-amyloid metabolism and improve cognition in a transgenic mouse model of Alzheimer's disease 24 h after single treatment

D Mengel[1,6], S Röskam[1,2,6], F Neff[1,3], K Balakrishnan[1], O Deuster[1,4], M Gold[1], WH Oertel[1], M Bacher[1,5], J-P Bach[1] and R Dodel[1]

There is evidence that naturally occurring antibodies directed against Aβ (nAbs-Aβ) have a role in Aβ-metabolism and Aβ-clearance. The presence of nAbs-Aβ leads to a reduction in amyloid fibrillation and thus a reduction in their toxicity. We investigated the effects of nAbs-Aβ in respect to oligomerization and used the Tg2576 transgenic mouse model in order to investigate the rapid effect with a single-dose (24 h) on oligomer breakdown and cytokine secretion along with immunohistochemical characterization of synaptic plasticity. nAbs-Aβ were able to reduce toxic oligomer concentration with an increase in Aβ-monomers. Cytokine secretion was significantly reduced. Synaptic plasticity was also improved after administration of nAbs. Finally, single treatment lead to a significant improvement in cognition. This study demonstrates the efficacy of nAbs-Aβ and presents evidence that several hallmarks of the disease are targeted by nAbs-Aβ.
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Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and affects ~20–30 million people worldwide. The histopathological hallmarks of AD are the occurrence of extracellular neuritic and non-neuritic senile amyloid plaques and intracellular neurofibrillary tangles, which are thought to trigger synaptic dysfunction, reduce synaptic density and eventually lead to neuronal cell death.[1,2] Recent evidence points to a crucial role of toxic Aβ oligomers rather than plaque formation in the development of this devastating disorder.[3,4]

Schenk and co-workers demonstrated that active immunization directed against β-amyloid (Aβ) reduced the plaque load considerably in an APP transgenic mouse model.[5] This groundbreaking discovery led to the development of a variety of approaches towards active immunization with Aβ as well as passive immunization. Passive immunization with murine monoclonal and, to a lesser extent, murine polyclonal Aβ-antibodies in APP transgenic mice, resulted in both reduction of Aβ burden and improvement of cognitive functions.[6,7] Previous studies from our group and from other investigators have recently detected antibodies against Aβ in the serum and consequently in human intravenous immunoglobulin preparations (IVIg); these naturally occurring autoantibodies (nAbs-Aβ) have been shown to inhibit Aβ toxicity and inhibit Aβ oligomerization *in vitro*.[8–10] Human IVIg are already being tested in clinical phase II/III trials, making them promising therapeutics for AD.[11,12] Further evaluation of their role in AD as well as their physiological importance needs to be clarified.[8,13] Recently, we were able to provide first evidence for a protective function of nAbs-Aβ on oligomer Aβ peptide toxicity and cognition in an animal model for AD after treatment for a 4-week period.[14] The aim of the present study is to further elucidate the mechanism of action of affinity-purified nAbs-Aβ 24 h after treatment *in vitro* and *in vivo* in an APP transgenic mouse model for AD.

Materials and methods

Purification of nAbs-Aβ. For a detailed description see previous publications.[14,15] Briefly, we used purified human intravenous IgG (Octagam 5%, which was kindly provided by Octapharma AG, Lachen, CH, Switzerland) for the isolation of nAbs-Aβ. For the following isolation steps this preparation was mixed with an equal volume of phosphate-buffered saline (PBS) and loaded directly onto an affinity column. As the $Aβ_{1-40}$ sequence contains internal lysine residues, which might lead to side reactions in immobilization procedures using amino groups, a specific affinity column was prepared using a cysteine residue attached to the Aβ-N-terminus to ensure homogeneous orientation of peptide molecules on the column support by immobilization through cysteinyl-S-thioether linkage. The azlactone-activated support contains an iodoacetyl group (Ultralink; Perbio, Bonn, Germany) at the end of a hexadecyl-spacer group, which reacted with the

[1]Department of Neurology, Philipps-University Marburg, Marburg, Germany; [2]Department of Animal Physiology, Philipps-University, Marburg, Germany; [3]Institute of Pathology, Helmholtz Zentrum, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), München, Germany; [4]IZKS-Mainz, University Medical Center, Mainz, Germany and [5]Institute of Immunology, Philipps-University, Marburg, Germany
Correspondence: R Dodel, Department of Neurology, Philipps-University Marburg, Baldingerstrasse, 35041 Marburg, Germany.
E-mail: dodel@med.uni-marburg.de
[6]These authors contributed equally to this work.
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cysteinyl-sulfhydryl group to yield a stable thioether linkage in order to reduce steric hindrance and provide maximum binding capacity of the antibodies. For covalent attachment of the Cys-A$\beta_{1\text{-}40}$, 3.7 mg of peptide was dissolved in 50 mM Tris and 5 mM EDTA-Na coupling buffer (pH 8.5) to a final concentration of 0.37 mg ml^{-1}. The solution was added to 1 ml of drained ultralink-iodoacetyl gel and the coupling reaction was performed for 1 h at room temperature under gentle mixing, followed by 30 min reaction time without mixing. An aliquot of 0.5 ml of the Cys-A$\beta_{1\text{-}40}$ coupled support was packed into a column (2.5 ml, MoBiTec, Goettingen, Germany) allowing the solution to drain. The column was washed with 3 ml of coupling buffer, and non-specific binding sites on the gel were blocked for 2×45 min with 1 ml of 50 mM L-Cysteine-HCl in coupling buffer. Subsequently the column was washed with 5 ml of 1 M NaCl and 5 ml of 0.1 M Na-phosphate, 0.15 M NaCl (pH 7.2) and stored at 4 °C. The gel support (0.5 ml) was transferred into a 15 ml falcon vial using 5 ml PBS and mixed with 5 ml IVIg. After gentle shaking overnight at 4 °C, this suspension was transferred to the column using the effluent to completely rinse the matrix back into the column. The column was washed eight times with 10 ml of PBS followed by two wash cycles with 10 ml ultrapure water.

The bound antibodies were eluted from the column with 10×0.5 ml 0.1 M glycine buffer, pH 2.8. Each fraction was collected in a microreaction tube containing 35 µl 1 M Tris-HCl, pH 9. The flow-through of this isolation procedure, which contains IgG depleted of nAbs-Aβ, was also collected and was used as a control in the respective experimental settings.

To maintain the integrity of the antibodies, a neutral pH was adjusted immediately after elution by adding the appropriate amount of Tris-HCl or glycine buffer. To regenerate the column for further use, the column was washed once with 10 ml 10 mM Na-phosphate buffer pH 6.8, followed by two wash cycles with 10 ml of PBS containing 1 M NaCl and finally two wash cycles with 10 ml PBS.

Antibody concentrations in the elution fractions were determined by the Micro BCA Protein Assay Kit method (Pierce; Perbio, Bonn, Germany). The stock solution of 2 mg ml^{-1} of bovine albumin supplied within the Micro BCATM Kit was used to prepare fresh standard dilutions within the range 40–0.5 µg ml^{-1}. The antibodies were eluted between fractions $1-6$, with the highest concentrations in fractions 1 and 3. For quantification of each set of ten elutions, fresh albumin standard dilutions were prepared. Results were read at 562 nm with an ELISA reader. Detailed affinity studies on these isolated IgG have been performed in a recent communication.[14]

Antibodies and chemicals. The following antibodies were used: antibodies against Aβ (clone 6E10, Gentaur, Brussels, Belgium); species-specific horseradish peroxidase-conjugated secondary antibodies (Cell signaling, Danvers, MA, USA); All remaining chemical compounds were obtained from Sigma-Aldrich (Hamburg, Germany) and Carl Roth (Karlsruhe, Germany) if not otherwise stated.

Aβ oligomerization. Toxic oligomers were synthesized according to a modified protocol by Kayed *et al.*[16] Briefly, 300 µg A$\beta_{1\text{-}40}$/A$\beta_{1\text{-}42}$ were dissolved in 90 µl hexafluoroisopropanol and 210 µl deionized water and diluted with 900 µl 100 mM NaCl, 50 mM Tris, pH 7.4 at 25 °C. The solution was stirred for 48 h at 37 °C. The tubes were weighed and the evaporated alcohol was replaced with 100 mM NaCl, 50 mM Tris, pH 7.4. The Aβ concentration of this oligomer-enriched solution is 56 µM. To study whether nAbs-Aβ interfere with Aβ oligomers, 56 µM oligomer-enriched Aβ was incubated in the presence of 0.3 µM nAbs or solvent (Tris-buffered saline) for 24 h at 37 °C. The effect of nAbs-Aβ on Aβ oligomerization and fibrillization was studied by adding 2 µM nAbs-Aβ or solvent (tris-buffered saline) to 56 µM of solubilized Aβ before oligomerization.

Western blot. Three hundred nanograms Aβ (PSL GmbH, Germany) were loaded on precasted NuPage novex 4–12% Bis-Tris gels (Novex-system, Invitrogen, Darmstadt, Germany). Proteins were transferred onto nitrocellulose membranes (Whatman, Dassel, Germany) using XCell II blot (Invitrogen). Membranes were blocked overnight at 4 °C with Roti Block (Carl Roth) processed according to standardized procedures.

To distinguish between different Aβ isoforms, we used a protocol by Klafki *et al.*[17] Aβ standard samples for A$\beta_{1\text{-}38}$/A$\beta_{1\text{-}40}$/A$\beta_{1\text{-}42}$ were purchased from Bachem, Switzerland. 100 µg of mice brain lysate were loaded per lane.

Soluble APPα and Aβ detection in cerebrospinal fluid (CSF). Soluble APPα (sAPPα) and A$\beta_{1\text{-}x}$ concentrations in CSF were determined by using sAPPα and A$\beta_{1\text{-}x}$ ELISA-kits (IBL, Hamburg, Germany).

Cytokine and Aβ measurement in brain lysates and serum samples. Mice brains were lysed in T-PER lysis buffer (Pierce, Rockfort, IL, USA) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). Cytokine concentrations of brain lysates and serum samples were measured with specific ELISA-kits (R&D, Wiesbaden, Germany) according to the manufacturer's instructions. Measurements were performed for IL-1β, IL-6, TNF-α and INF-γ. Aβ concentrations were analyzed by A$\beta_{1\text{-}x}$ ELISA (IBL, Hamburg, Germany).

Animals. Twenty to 22-month-old as well as 27 to 30-month-old heterozygous adult female Tg2576 mice expressing mutant APP$_{SWE\ (695(K670N,M671L)}$[18] under the control of the hamster prion promoter on a hybrid C57Bl/6xSJL background were used. Age- and gender-matched non transgenic wild-type mice (WT) were used as control. Tg2576 and control mice were randomly assigned to groups of 5–6, independent of genotype and treatment on a 12 h light-dark schedule (lights on 07:00–19:00). They had free access to tap water, were fed *ad libitum* and kept under standard conditions. Tg2576 mice weighed between 30–40 g at the beginning of the experiments. Behavioral experiments were conducted during the light period between 12:00–18:00 h. Sample sizes of the groups were as follows: transgenic (Tg) control $n=11$, Tg nAbs-Aβ $n=12$, WT $n=11$ for 20 to 22-month-old mice. Tg control $n=9$, Tg nAbs-Aβ $n=12$ and WT $n=6$ for 27 to 30-month-old mice.

All animal procedures were approved by the office of the federal state authority of Hessen and the Institutional Animal Care and Use Committee of the University of Marburg.

Administration of nAbs-Aβ to mice. Mice were treated intraperitoneally with nAbs-Aβ (400 μg dissolved in 0.2 ml of PBS) or vehicle (0.2 ml PBS) 24 h before assessing cognition or killing for brain tissue and CSF sampling. To ensure that the treatment period is 24 h, two different groups of animals were treated for cognition experiments and tissue sampling, respectively. Transgenic animals treated with PBS are displayed as Tg Control, nAbs-treated mice as Tg + nAbs-Aβ. Wildtype mice are referred to as WT.

Visuospatial learning task. The object location test (OLT) is based on the spontaneous tendency of rodents to explore an object moved to a new location more often than an object located at the familiar position.[19] In the OLT, animals are introduced to two identical objects in an experimental apparatus and, after a delay, are exposed again to the same two objects, while one of them has been displaced to a new location. Animals that remember the previous exposure spontaneously spend more time exploring the object in the new position.

For analysis of OLT, the amount of time spent investigating the object located in the familiar position in the training trial and on the object which had been displaced to a new location in the test trial was recorded for each mouse. In addition, a discrimination ratio (time spent on the object in the novel location vs the total time spent exploring both objects in the test trial) was calculated for each mouse. OLT was reflected by more time interacting with the object in

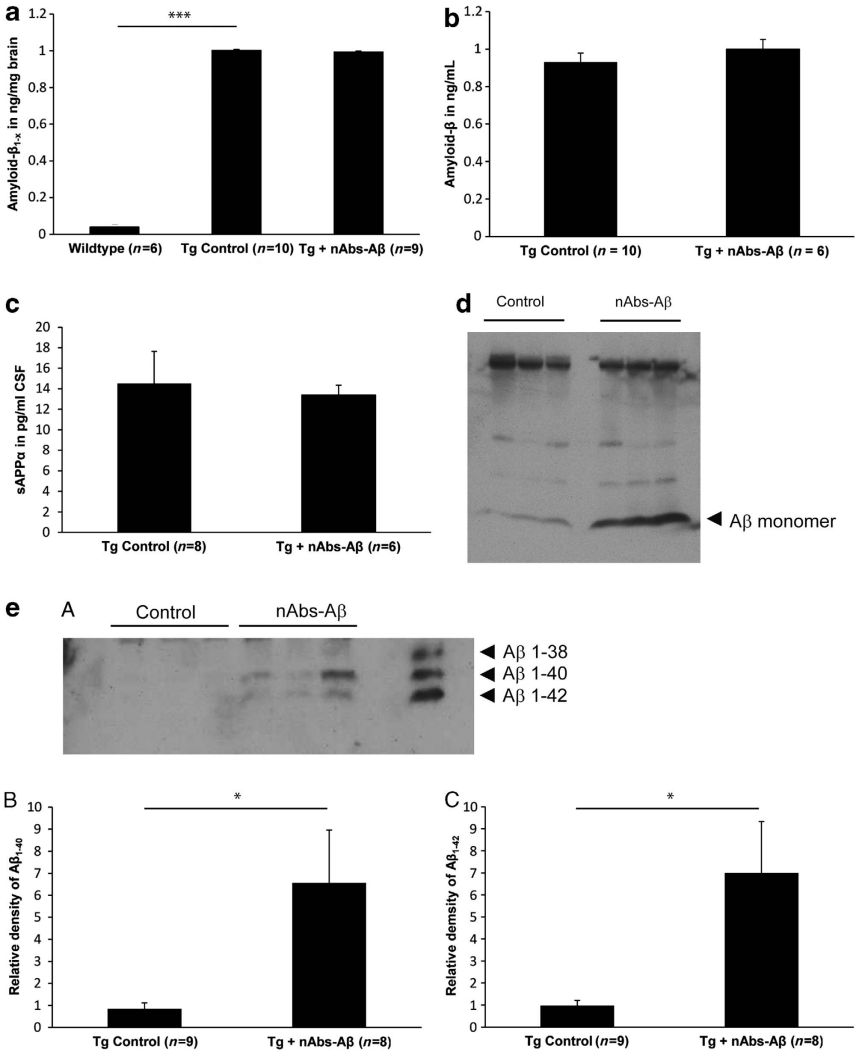

Figure 1 nAbs-Aβ lead to oligomer breakdown *in vivo* in 27 to 30-month-old Tg2576 mice. Total Aβ concentrations in brain lysates (**a**) and CSF (**b**) of treated and control mice are constant. Animals were treated with 400 μg nAbs or vehicle (PBS) for a duration of 24 h. Measurements were performed using ELISA kits. (**c**) Soluble sAPPα is also unchanged in animals treated with nAbs-Aβ compared both to vehicle treated and control animals. (**d**) Brain lysates were analyzed using western blot (anti Aβ clone 6E10 monoclonal antibody). Single treatment of transgenic animals with nAbs-Aβ leads to an increase in monomeric Aβ compared with control animals. (**e**) Further characterization of these monomers demonstrated that $Aβ_{1-40}$ is most abundant, with $Aβ_{1-42}$ being less abundant, whereas hardly any $Aβ_{1-38}$ can be detected (see A). Densitometric analysis for $Aβ_{1-40}$ (B) and $Aβ_{1-42}$ (C) showed a sevenfold increase in the treatment group, respectively (anti-Aβ clone 6E10 monoclonal antibody was used to detect Aβ).

the novel location than with the object in the familiar position and a discrimination ratio above 0.5. Analysis of discrimination ratios was less subject to the influence of individual variability in contact times with objects and provided a measure of the extent of the discrimination between the novel and sample objects. Mice with a total investigation time less than 2 s were excluded from OLT due to reduced exploratory behavior.

Brain tissue and CSF sampling. Twenty-four hours after the injection, animals were anesthetized and CSF was obtained by puncture of the cisterna magna as previously described.[20] CSF was immediately frozen at $-80\,^{\circ}C$. After surgical isolation of mice brains, the cerebellum was removed and the remaining hemispheres bisected. One hemisphere was fixed in 4% neutral buffered formalin for histological analysis, whereas the remaining hemisphere was frozen $-80\,^{\circ}C$ for subsequent analysis.

Synapse detection using the Golgi-Hortega stain. The mouse brain samples were fixed and stained using the rapid Golgi-impregnation method as previously described.[21] Briefly, after fixation in 4% neutral buffered formalin, brains were immersed in darkness for 5 days in 3% potassium bichromate, with fresh solution every day. Excess potassium bichromate was whipped away when brain samples were transferred into 2% silver-nitrate solution for 3 days in darkness, with fresh solution every day. Samples were cut in 80 μm thick slices using a vibratome T1000 and mounted with gelatin. Pictures were taken immediately using a TE2000i Microscope from Nikon (Tokyo, Japan). Synaptic spines and axon length were detected manually using the NIS elements BR software (Kingston, UK). Data were exported to Excel (Microsoft, Redmond, WA, USA) for further statistical analysis.

Statistical analysis. All analyses were performed using the statistical software packages SigmaStat (Statcon, Witzenhausen, Germany) or SPSS15.0 for Windows (SPSS GmbH Software, Germany). All results are presented as the mean $\pm$ s.e. of the mean(s.e.m.).

The effect of acute immunization with nAbs-Aβ on OLT were analyzed using either one-way or two-way analysis of variance, followed by *post-hoc* Tukey's *t*-test for pair wise comparisons. Within-group *t*-test analysis was performed for each treatment group to show deficits in the object location memory. For all statistical comparisons, the following definitions were used: $P<0.05$(*), $P<0.01$(**) or $P<0.001$(***).

Results

Treatment with nAbs-Aβ increase soluble Aβ monomers. Treatment of 27 to 30-month-old Tg2576 mice with 400 μg nAbs-Aβ for 24 h had no influence on total $A\beta_{1-x}$ levels in brain lysates as well as in the CSF (Figures 1a and b). CSF sAPPα concentration was also not affected in nAbs-Aβ treated compared with control animals (Figure 1c). Interestingly, a considerable increase in soluble intracerebral Aβ monomers could be observed in treated animals compared with control animals, which were only treated with PBS (Figure 1d). Untreated transgenic animals only contained few soluble Aβ monomers in the brain, whereas a 24 h treatment with nAbs-Aβ lead to an increase in the fractions of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ (Figure 1e). Densitometric analysis revealed that treatment with nAbs-Aβ increased brain $A\beta_{1-40}$, $A\beta_{1-42}$ monomers by a factor of 7.2 ($\pm$ 2.4, $P<0.05$) and sevenfold ($\pm$ 2.3, $P<0.05$), respectively.

***In vitro* inhibition of Aβ oligomerization by nAbs-Aβ.** We performed *in vitro* experiments in order to further investigate the effect of nAbs-Aβ towards Aβ oligomers and evaluated the effect of nAbs-Aβ on *de novo* formation of Aβ oligomers using a western blot. Aβ was pretreated before oligomerization with 2 μM nAbs-Aβ. As can be seen in Figure 2a (A), there is a reduction in higher order oligomer formation following the treatment with nAbs-Aβ. Simultaneously, the amount of monomers, dimers and trimers increased following treatment with nAbs-Aβ. Densitometric analysis of the bands is shown in Figure 2a (B). The reduction in fibrils and higher order oligomers is statistically significant, as well as the observed increase in monomers and small oligomers such as dimers and trimers in the treatment group. Next, we analyzed the ability of nAbs-Aβ to break down oligomeric Aβ. Incubation of Aβ oligomers with nAbs-Aβ for 24 h showed no effect compared with solvent only (Figure 2b). Although, further treatment of Aβ oligomers for 24 h with either nAbs-Aβ or solvent showed a pronounced generation of oligomeric Aβ with a molecule size between 30 and 100 kDa due to a longer oligomerization process in comparison to the previous experiment.

Influence of nAbs-Aβ on proinflammatory cytokines in transgenic animals. As there was a reduction in oligomers *in vitro* following treatment with nAbs-Aβ and an improvement in cognition in the animal model after a single treatment, we evaluated proinflammatory cytokine concentrations in the brain homogenates of WT animals as well as 27 to 30-month-old Tg2576 mice treated with nAbs-Aβ. For all analyzed cytokines we were able to detect an upregulation in transgenic animals (Figure 3). IFN-γ was elevated by a factor of 3.6 compared with WT animals (Figure 3a)(6.10 ± 0.80 vs 21.54 ± 2.07, $P<0.001$), IL-1β levels (Figure 3b) in Tg2576 mice brain were increased 5.7-fold (2.97 ± 0.27 vs 17.28 ± 1.81, $P<0.001$), IL-6 (Figure 3c) was increased 2.6-fold (6.55 ± 0.19 vs 17.30 ± 1.77, $P<0.001$) and TNF-α (Figure 3d) showed a 2.4-fold increase (28.32 ± 0.57 vs 67.95 ± 6.90, $P<0.001$), respectively. Treatment of Tg2576 mice with nAbs-Aβ led to a significant reduction in the brain cytokine levels. IL-1β concentrations (Figure 3b) were reduced by 63% ($\pm$ 6%, $P<0.001$), IL-6 concentration (Figure 3c) was lowered by 64% ($\pm$ 7%, $P<0.001$), TNF-α levels (Figure 3d) were reduced by 65% ($\pm$ 3%, $P<0.001$) and IFN-γ levels (Figure 3a) were reduced by 60% ($\pm$ 3%, $P<0.001$).

Improvement of visuospatial learning in Tg2576 mice after single administration of nAbs-Aβ. Figure 5a shows the mean interaction time of the mice with the objects during

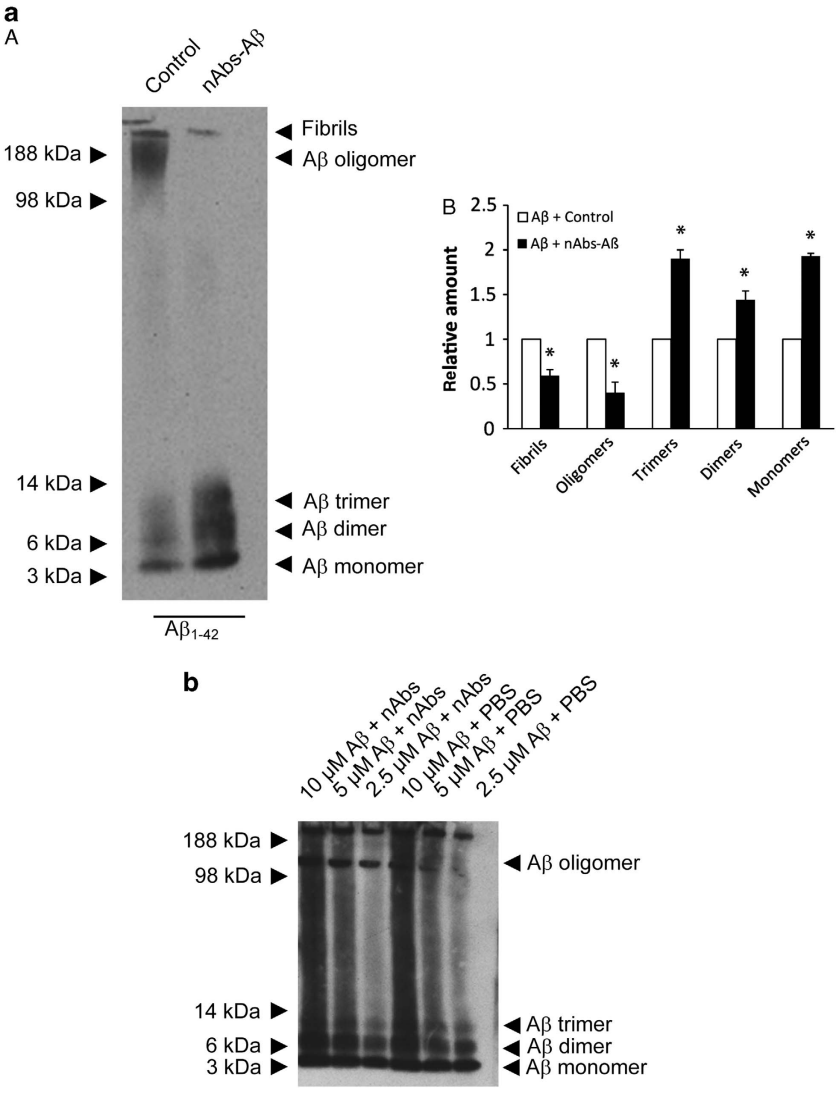

Figure 2 *In vitro* breakdown of Aβ oligomers. Further analysis was performed using western blot (**a**). Synthetic $A\beta_{1\text{-}42}$ oligomers were synthesized as described and 300 ng were loaded on the gel (A) Densitometric analysis of the bands (B) demonstrated a significant reduction in fibrils and oligomers with a concomitant increase in monomers in the treatment group compared with placebo. Incubation of oligomerized Aβ with nAbs-Aβ or solvent after the oligomerization process shows no effect. Shown are different concentrations of Aβ (2.5–10 μM) which are incubated with 0.3 μM nAbs-Aβ for 24 h. (**b**) Preincubation of Aβ with nAbs-Aβ before the oligomerization process reduces the generation of multimeric Aβ forms. As a result of this effect monomers and smaller oligomers are increased when Aβ is pretreated with nAbs-Aβ. Controls were incubated with PBS only (in both blots anti Aβ clone 6E10 monoclonal antibody was used to detect Aβ).

the training trial. No significant differences were found between groups regarding object exploration, indicating intact exploratory behavior. A significant result was found concerning main effect of object ($F_{(1,72)} = 15.13$; $P < 0.001$) and interaction between object and treatment ($F_{(2,72)} = 4.22$; $P < 0.05$) during the test trial. nAbs-Aβ treated 22-month-old Tg2576 mice ($P < 0.001$) and WT control mice ($P < 0.01$) interacted significantly longer with the object in the novel location compared with PBS treated animals (Figure 5b). A discrimination ratio above 0.5 reflects object location memory and between-group analysis revealed that 22-month-old Tg2576 mice immunized with nAbs-Aβ ($P < 0.001$) and WT mice ($P < 0.001$) showed similar relative preferences for the object located to a novel position compared with PBS-treated Tg2576 mice (Figure 5c). Single immunization with nAbs-Aβ significantly ameliorated impairments in visuospatial learning in Tg2576 mice.

nAbs-Aβ treatment influences synaptic plasticity. From the data shown above we hypothesized that nAbs-Aβ may also improve synaptic plasticity, which is affected by cytokines as well as toxic Aβ oligomers.

To determine the influence of nAbs-Aβ onto synaptic plasticity (formation and degradation of dendritic spines), parts of the brains were impregnated with silver according to the protocol established by Golgi, Cajal and Hortega. Spiny neurons in the visual cortex were identified, and after dendrites of the second level had been pegged, axon length and synaptic spines were determined. Twenty-two-month-old Tg2576 animals treated with nAbs-Aβ displayed significantly

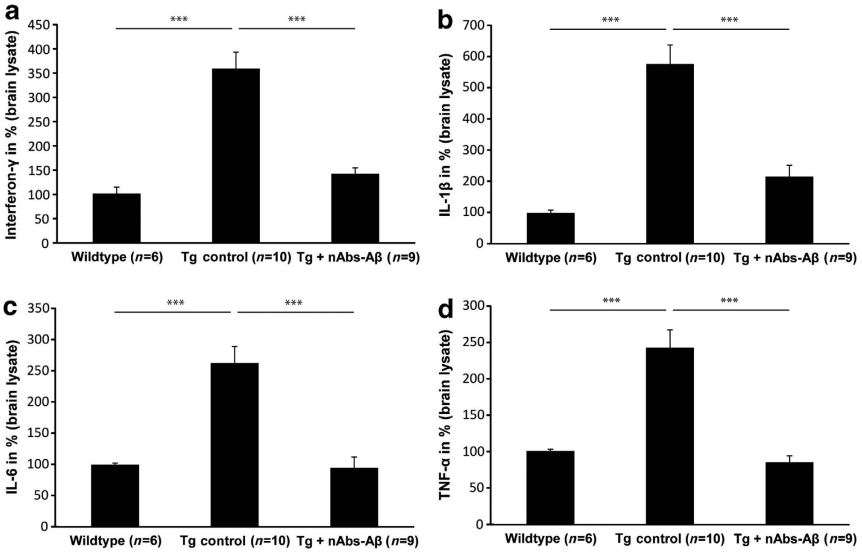

Figure 3 Cytokine changes in response to nAbs treatment in 27 to 30-month-old Tg2576 mice. Cytokines were measured using standard ELISA-assays. Measurements were performed for IFN-γ (**a**), IL-1β (**b**), IL-6 (**c**) and TNF-α (**d**) in brain lysates. For all four cytokines, there is a highly significant increase when comparing WT and untreated animals. Treatment with 400 μg nAbs-Aβ for 24 h leads to a reduction to background secretion in WT animals.

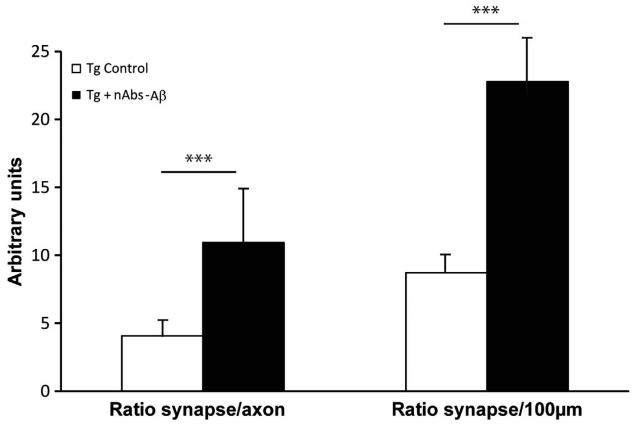

Figure 4 Treatment with nAbs-Aβ lead to restoration of synaptic densities in 22-month-old Tg2576 mice. Synapses were determined as described in the Materials and Methods section. Synapse numbers in relation to the axon number and the proportion of synapses per 100 μm axon length is provided. A significant increase in synapses is readily visible in Tg2576 mice following treatment with nAbs-Aβ in contrast to PBS.

higher mean synapse numbers than those treated with PBS. The relation of synapses per axon was significantly higher in the Tg2576 animals treated with nAbs-Aβ comparedwitho the PBS-treated group (Figure 4). A similar significant result was obtained calculating the mean synapse number in relation to 100 μm axon length (Figure 4).

Discussion

This study investigated the biochemical changes and behavioral effects of a single passive immunization with human nAbs-Aβ 24 h before cognitive testing in a transgenic mouse model for AD. There is increasing evidence for a major role of toxic oligomers in the development of AD.[22] According to a study by McLaurin *et al.*,[23] monoclonal antibodies against Aβ were able to interfere with fibrillization and oligomerization processes of Aβ. We therefore investigated the role of nAbs-Aβ in a transgenic animal model of AD, where we were able to show that total brain and CSF $Aβ_{1-x}$ concentration was unaffected by the treatment with nAbs-Aβ. Subsequent western blotting showed an increase in monomeric Aβ following administration of nAbs-Aβ. There are several possible explanations for this observation. It may be possible that nAbs-Aβ in general lead to an increased production of Aβ. The main argument against this hypothesis is the observation that total brain as well as CSF Aβ concentrations were constant. In addition, Yamada *et al.*[24] were able to show that secretase activity was not influenced by antibody treatment with the monoclonal antibody m266. Another possibility is that nAbs-Aβ are able to break oligomers or to dissolve Aβ out of senile plaques. Previous data as well as our *in vitro* data argue against this possibility.[14] Recently, we investigated the application of nAbs-Aβ in a transgenic animal model. In this study, no clearance of amyloid plaques could be observed. In addition, these data point towards a role of nAbs-Aβ to interfere with Aβ peptide toxicity and their ability to oligomerize. Therefore, oligomerization and fibrillization may be inhibited by nAbs-Aβ. In our study we demonstrated that nAbs-Aβ are unable to break down oligomeric as well as fibrillar Aβ *in vitro.* However, *de novo* oligomerization to higher aggregated Aβ ($n>3$) and fibrillization of Aβ could be inhibited by coincubation with nAbs-Aβ. However, we could also observe significant increase in small Aβ oligomers as dimers and trimers following incubation with nAbs-Aβ. Evidence has accumulated that these forms are the toxic aggregation form of Aβ.[25,26] Though, Lesne and others could show, that higher aggregated Aβ, such as oligomers of a size of 56 kDa, are the toxic species that impair memory in the Tg2576 mice model.[27] Sherman *et al.*[28] could also detect these Aβ*56 oligomers in

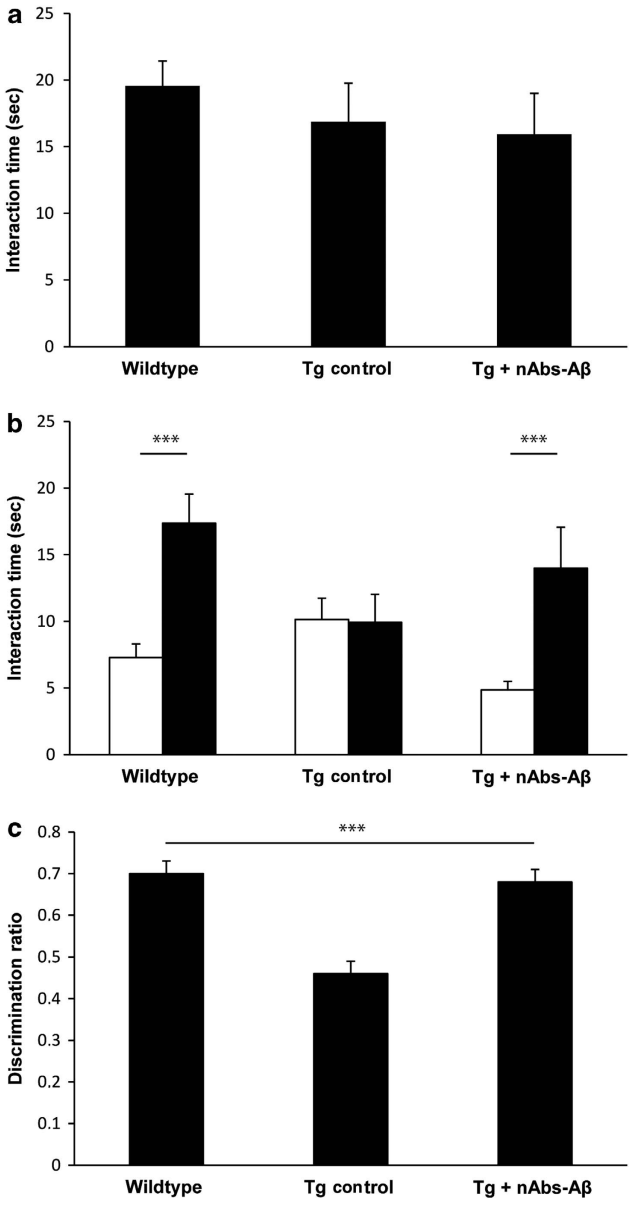

Figure 5 Single immunization with nAbs-Aβ leads to rapid improvement of visuospatial learning and memory in 22-month-old Tg2576 mice after 24 h. Object location memory in 20 to 22-month-old Tg2576 nAbs-Aβ treated ($n = 12$), Tg2576 Control ($n = 11$) and WT mice ($n = 11$). (**a**) No significant differences were found between groups regarding object exploration, indicating intact exploratory behavior during the training trial. (**b**) Tg2576 nAbs-Aβ treated and WT, but not Tg2576 Control mice, showed a significantly increased interaction time with the object moved to a novel position compared with the object in the familiar location (**$P < 0.01$, ***$P < 0.001$) during the test trial. (**c**) A discrimination rate above 0.5 reflected preference for the object in the novel position, and Tg2576 nAbs-Aβ treated and WT, but not Tg2576 Control mice, showed a similar preference towards the relocated object (***$P < 0.001$). A within-group *t*-test analysis revealed an object location memory deficit in Tg2576 control mice. Tg2576 nAbs-Aβ treated mice showed a significant improvement of visuospatial learning and memory after 24 h using the object location memory test. Data presented for all groups are means ± s.e.m.

brain tissues. Therefore, so far it can be concluded that there is ongoing discussion about which aggregation forms in particular are causative for memory impairment in mouse models of Alzheimers disease as well as in humans. A similar mode of action of antibody treatment in transgenic mice was suggested by Yamada *et al.*[24] At the same time, total brain Aβ levels remained constant. The authors concluded that anti-Aβ antibodies are able to bind soluble Aβ in the brain and thus preventing the accumulation of toxic multimeric forms. This is in line with our observation. Our data also point to a binding of nAbs-Aβ to monomers and small oligomeric forms and a subsequent sequestration, which precludes the generation of higher oligomeric species and fibrils. We therefore assume that one important mechanism leading to cognitive improvement in this transgenic animal model is the inhibition of Aβ oligomerization. An important question is the fate of the intracerebral antibody-bound Aβ. Winkler *et al.*[29] observed a rapid increase in serum Aβ in contrast to no changes in intracerebral Aβ as well as CSF Aβ levels levels following treatment with monoclonal anti-Aβ antibodies. Of note, Winkler *et al.*[29] suggested a peripheral mode of action by antibody binding to serum Aβ and subsequent stabilization. Though, Winkler *et al.*[29] could also observe a strong cerebral amyloid-plaque binding following infusion with anti-Aβ antibodies. Interestingly, we could also detect an effect of nAbs-Aβ on intracerebral Aβ. In addition to a sequestration of Aβ in plasma referring to the peripheral sink hypothesis as postulated by DeMattos *et al.*[30,31] our study points towards a penetration of antibodies into the CNS and a direct intracerebral activity. This is also in line with the aforementioned study by Yamada *et al.*,[24] and our own study showing a cerebral accumulation of human nAbs-Aβ in the APP23 model.[32] An analysis of serum Aβ was not part of this study. To address the question of the fate of the increased brain monomeric Aβ following treatment with nAbs-Aβ an analysis of serum levels should be performed in a further study.

Furthermore, we analyzed the influence of nAbs-Aβ on cytokine concentrations in Tg2576 mice. Compared with WT littermates, there was an increase in the proinflammatory cytokines IL-1, IL-6, TNF-α and IFN-γ in transgenic animals. nAbs-Aβ treatment led to a reduction in cytokine release in our animal model of AD. Proinflammatory cytokines are known to have an important role in AD.[33] Therefore, it is most likely that cytokines are important in the maintenance of a homeostatic environment and required for proper functioning of complex neuronal circuits. Interestingly, cytokine concentrations, localization as well as timing of its secretion seem to be of importance.[34,35] Our data show a reduction of cytokine concentration in APP transgenic mice similar to those seen in WT littermates. It seems likely that nAbs-Aβ therapy helps to restore the physiological cytokine environment. We argue that inhibition of Aβ oligomerization is partially responsible for this effect. However, a direct effect, of nAbs-Aβ on proinflammatory cytokine levels cannot be ruled out. Luo *et al.*[36] could show a direct effect of intravenous Ig on intracerebral IL-6 and interferon-γ levels in a mouse model of epilepsy.

It is known that Aβ oligomers lead to synaptic dysfunction.[37] Recently, it was reported that a single dose of passive immunization against Aβ led to a remarkable improvement of synaptic structures within hours, and that this effect could last for 30 days.[38] We observed a modulation of synaptic plasticity

following administration of nAbs-Aβ. There are several explanations for the described improvement in synaptic plasticity. Our previous study showed a strong binding of nAbs-Aβ to Aβ oligomers and a protective effect towards Aβ-oligomer induced neurotoxicity. A prevention of Aβ-dependent impaired synaptic transmission by antioligomeric antibodies has been recently reported by Hillen *et al.*[39] Additionally, nAbs-Aβ prevent the generation of oligomers by inhibiting aggregation and could subsequently reduce a synaptotoxic influence of Aβ oligomers. As we could also observe a reduction of intracerebral proinflammatory cytokines in treated animals, this may also contribute to the restoration of synaptic plasticity.

Finally, we observed impaired cognition in Tg2576 mice with deficits in object location memory; acute passive immunization with nAbs-Aβ reversed spatial memory impairments in Tg2576 mice. A number of studies are published showing beneficial effects of active and passive immunization in APP transgenic mouse models for AD using different spatial learning and memory tests (for review, see Röskam *et al.*[7]). A range of passive immunization strategies were found to be as effective as active vaccination on spatial learning and memory experiments.[40] Treatment with 2286 (a mouse monoclonal antihuman $A\beta_{28-40}$ antibody) in Tg2576 mice reversed impairments in the radial arm water maze test.[41] Triple transgenic TgAD mice immunized with a monoclonal $A\beta_{1-17}$ antibody (1560) or 20.1 (a monoclonal $A\beta_{1-8}$ antibody) were found to ameliorate deficits seen in the T-maze and MWM tests.[42,43]

In summary, we provide further evidence for a potential role of naturally occurring antibodies in the context of AD. The significant therapeutic effect of an acute passive immunotherapy with nAbs-Aβ on object location memory in old-aged Tg2576 mice one day before testing is based on several mechanisms. Reversal of synaptic dysfunction and subsequent restoration of synaptic communication in the neural systems, modulation of proinflammatory cytokine release as well as a reduction of toxic oligomers are probably the main mechanisms responsible for the observed cognitive improvements in Tg2576 mice.

Early studies by our group have demonstrated the effective use of IVIg, which contain nAbs-Aβ, in patients with AD.[44] Similar results were obtained by Relkin *et al.*[12] Therefore, isolation of naturally occurring antibodies from IVIg preparations is a first step in order to prove their efficacy. In a second step, it is essential to understand their physiological role, so that finally, these can be heterologously expressed or chemically synthesized in a large scale. Further research is therefore required to investigate the role of nAbs-Aβ, but we consider their high potential in the treatment of AD.

Conflict of interest

Dr Bacher, Dr Balakrishnan, Dr Dodel hold patents in respect to naturally occurring autoantbodies. All other authors have no conflict of interest concerning the contents of the article.

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GLIA

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Activation of *KCNN3*/SK3/K_{Ca}2.3 Channels Attenuates Enhanced Calcium Influx and Inflammatory Cytokine Production in Activated Microglia

AMALIA M. DOLGA,[1]* TILL LETSCHE,[1,2] MAIKE GOLD,[2] NUNZIANNA DOTI,[1,3] MICHAEL BACHER,[2] NIPAVAN CHIAMVIMONVAT,[4] RICHARD DODEL,[2] AND CARSTEN CULMSEE[1]

[1]*Institute of Pharmacology and Clinical Pharmacy, Philipps University of Marburg, 35032, Marburg, Germany*
[2]*Department of Neurology, Philipps University of Marburg, 35043, Marburg, Germany*
[3]*Institute of Biostructures and Bioimaging–CNR, 16–80131, Naples, Italy*
[4]*Department of Medicine, University of California, Davis, California*

KEY WORDS
potassium *KCNN*/SK/KCa2 channels; CyPPA; calcium homeostasis; microglia; cytokines

ABSTRACT
In neurons, small-conductance calcium-activated potassium (*KCNN*/SK/K_{Ca}2) channels maintain calcium homeostasis after *N*-methyl-D-aspartate (NMDA) receptor activation, thereby preventing excitotoxic neuronal death. So far, little is known about the function of *KCNN*/SK/K_{Ca}2 channels in non-neuronal cells, such as microglial cells. In this study, we addressed the question whether *KCNN*/SK/K_{Ca}2 channels activation affected inflammatory responses of primary mouse microglial cells upon lipopolysaccharide (LPS) stimulation. We found that *N*-cyclohexyl-*N*-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine (CyPPA), a positive pharmacological activator of *KCNN*/SK/K_{Ca}2 channels, significantly reduced LPS-stimulated activation of microglia in a concentration-dependent manner. The general *KCNN*/SK/K_{Ca}2 channel blocker apamin reverted these effects of CyPPA on microglial proliferation. Since calcium plays a central role in microglial activation, we further addressed whether *KCNN*/SK/K_{Ca}2 channel activation affected the changes of intracellular calcium levels, $[Ca^{2+}]_i$, in microglial cells. Our data show that LPS-induced elevation of $[Ca^{2+}]_i$ was attenuated following activation of *KCNN2*/3/K_{Ca}2.2/K_{Ca}2.3 channels by CyPPA. Furthermore, CyPPA reduced downstream events including tumor necrosis factor alpha and interleukin 6 cytokine production and nitric oxide release in activated microglia. Further, we applied specific peptide inhibitors of the *KCNN*/SK/K_{Ca}2 channel subtypes to identify which particular channel subtype mediated the observed anti-inflammatory effects. Only inhibitory peptides targeting *KCNN3*/SK3/K_{Ca}2.3 channels, but not *KCNN2*/SK2/K_{Ca}2.2 channel inhibition, reversed the CyPPA-effects on LPS-induced microglial proliferation. These findings revealed that *KCNN3*/SK3/K_{Ca}2.3 channels can modulate the LPS-induced inflammatory responses in microglial cells. Thus, *KCNN3*/SK3/K_{Ca}2.3 channels may serve as a therapeutic target for reducing microglial activity and related inflammatory responses in the central nervous system.

INTRODUCTION

Microglial cells are the immune competent cells in the central nervous system originating from the peripheral mesodermal tissue during early postnatal development (Chan et al., 2007). Under physiological conditions, microglial cells display ramified morphology that is regarded as the resting phenotype (Kettenmann et al., 2011). During pathophysiological states, such as brain trauma, cerebral ischemia, or in neurodegenerative diseases, microglial cells become activated and change into a macrophage-like phenotype. Besides these pronounced morphological changes, activated microglia display increased proliferation rate, migration to the site of injury, increase in nitric oxide (NO) production, and release of several cytokines and chemokines (Rupalla et al., 1998).

Intracellular calcium $[Ca^{2+}]_i$ controls the microglial activity state and acts as a central element in microglial activation (Hoffmann et al., 2003). Ca^{2+}-permeable receptor-operated channels and Ca^{2+}-permeable store-operated channels are the major responsible for increases in $[Ca^{2+}]_i$ and related signaling pathways (Moller, 2002). Microglial receptors associated with calcium signaling and microglial activation pathways are multifaceted and include receptors for chemokines, complement factors, acetylcholine, endothelin, and purines (Kettenmann et al., 2011). In microglia, increases in $[Ca^{2+}]_i$ are necessary for developing full cytokine induction (Hoffmann et al., 2003). Furthermore, in human microglia, cytokines promote Ca^{2+} influx (Goghari et al., 2000), which, in turn, might further activate microglia and increase cytokine production in a positive feedback loop manner.

It was previously reported that $[Ca^{2+}]_i$-dependent signaling is tightly associated with Ca^{2+}-dependent potassium channels (Dolga et al., 2011; Stocker, 2004). For example, Ca^{2+}-dependent potassium channels, such as large conductance Ca^{2+}-activated potassium (BK_{Ca}) channels and small/intermediate-conductance Ca^{2+}-activated

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Amalia M. Dolga and Till Letsche contributed equally to this work.

Correspondence to: Amalia M. Dolga, Institut für Pharmakologie und Klinische Pharmazie, Fachbereich Pharmazie, Philipps-Universität Marburg, Germany; Karl-von-Frisch-Straße 1, 35032 Marburg, Germany. E-mail: dolga@staff.uni-marburg.de

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potassium (*KCNN1*-3/SK/IK/$K_{Ca}2$) channels have been linked to microglial activation processes (Bordey and Spencer, 2003; Schlichter et al., 2010). In the central nervous system, *KCNN1*/SK1/$K_{Ca}2.1$, *KCNN2*/SK2/$K_{Ca}2.2$, *KCNN3*/SK3/$K_{Ca}2.3$, and *KCNN4*/SK4/$K_{Ca}3.1$ channels are widely expressed and functional (Stocker, 2004). In microglial cells, SK/$K_{Ca}2$ channels may be involved in microglial respiratory burst and related production of toxic oxygen radicals and NO. For example, apamin, a blocker of all *KCNN*/$K_{Ca}2$ channel subtypes, was able to inhibit the respiratory burst in microglia (Khanna et al., 2001). In our study, we aimed to differentiate the potential contribution of each particular *KCNN*/SK/$K_{Ca}2$ channel subtype to microglial activation by using specific *KCNN*/SK/$K_{Ca}2$ channel inhibitory peptides.

Since increases of $[Ca^{2+}]_i$ potentiate microglial activation and contribute to full cytokine induction (Hoffmann et al., 2003), and *KCNN*/SK/$K_{Ca}2$ channels modulate calcium signaling (Dolga et al., 2011), we study the influence of *KCNN*/SK/$K_{Ca}2$ channels on microglial calcium homeostasis and its impact on lipopolysaccharide (LPS)-induced microglial activation. We investigate particularly the role of extracellular calcium and *KCNN2/3*/$K_{Ca}2.2$/$K_{Ca}2.3$ channels in microglial activation, associated morphological changes, and cytokine production. We explore *KCNN2/3*/$K_{Ca}2.2$/$K_{Ca}2.3$ channel pathways using the positive modulator *N*-cyclohexyl-*N*-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine (CyPPA) (Hougaard et al., 2007) in combination with specific inhibitory peptides for each of the *KCNN*/SK/$K_{Ca}2$ channel subtypes (Tuteja et al., 2010). Using these specific inhibitory peptides we revealed that *KCNN3*/SK3/$K_{Ca}2.3$ channel activation was required to inhibit inflammatory responses of primary microglial cells after exposure to LPS. These results expose *KCNN3*/SK3/$K_{Ca}2.3$ channels as potential therapeutic targets to block inflammatory responses of microglia in neurological diseases.

Abbreviations

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
GFAP	Glial fibrillary acidic protein
EDTA	Ethylenediaminetetraacetic acid
DAPI	4′,6-diamidino-2-phenylindole
EGTA	ethylene glycol tetraacetic acid
RT-PCR	Reverse transcription polymerase chain reaction
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
NMDAR	N-Methyl-D-aspartate receptor
BKCa channels	large conductance Ca^{2+}-activated potassium channels
[Ca2+]i	intracellular calcium
CI	cell index values
CyPPA	*N*-Cyclohexyl-*N*-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine
DMEM	Dulbecco's modified Eagle containing-calcium medium
DMSO	Dimethyl sulfoxide
FCS	fetal calf serum
*I*CRAC currents	Ca^{2+}-activated Ca^{2+} current
*I*DRK currents	voltage-gated and delayed rectifying potassium currents
*I*IRK currents	inward rectifying potassium currents
IL-6	interleukin 6
KATP channels	ATP-sensitive potassium channels
KCNN/SK/IK/KCa2 channels	Small/intermediate-conductance Ca^{2+}-activated potassium channels
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase protein
MCAo	middle cerebral artery occlusion
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NMDA	*N*-methyl-D-aspartate
NCIti	normalized cell index
NO	nitric oxide
OD	optical density
P2X(7) receptor	ATP-gated purinergic receptors
PFA	paraformaldehyde
TNF-α	tumor necrosis factor alpha
TRAM-34	triarylmethane-34
TRPM4 channel	Transient receptor potential cation channel subfamily M member 4.

MATERIALS AND METHODS

Primary Microglia Culture

Microglia cultures were prepared as previously described (Saura et al., 2003). Briefly, brains were removed from 1- to 3-day-old C57Bl/6 pups, minced, dissociated for 15 min in 1 mg/mL trypsin, and then cells were cultured in Dulbecco's modified Eagle containing-calcium medium (DMEM, PAA Laboratories, Cölbe, Germany) consisting of Hams F12 (50/50), supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. After 2 days of *in vitro* cultivation, the growth medium was completely replaced by fresh medium. After 10–14 days, flasks were mechanically shaken for 60 min, 150 rpm to yield microglia in the supernatant, which were sub-cultured into uncoated 96-well plates (15,000–17,000 cells/well). They were kept in 30% astrocyte conditioned medium and 70% fresh DMEM/F12 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine for 1-2 days to achieve microglia in a quiescent state. For all experiments, primary microglial cells were used only for the first and second passage. For experiments with DMEM without calcium, we have used DMEM (no. E15-078, PAA Laboratories) containing high glucose (4.5 g/L), HEPES (6 g/L), and sodium pyruvate (110 mg/L); and supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Primary mouse microglia were = 95% pure, as verified by labeling with F4/80 (BioLegend, San Diego, CA, no. 122601) and GFAP antibodies (Cell Signaling, no. 2118). The compounds CyPPA (Hougaard et al., 2007), EDTA, LPS, and apamin (Stocker, 2004) were obtained from Sigma, Deisenhofen, Germany. All compounds otherwise indicated were also purchased from Sigma (Deisenhofen, Germany).

LPS and Drug Treatment

Primary microglial cells were shaken off mixed glial cultures and plated in a density of 15,000–17,000 cells/well in 96-well plates overnight. Media was then changed to fresh DMEM/F12 (50/50) containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Microglia were left non-stimulated or activated with 200 ng/mL LPS (Sigma, Deisenhofen,

Germany) in the presence or absence of different concentrations of CyPPA (10–75 μM) or apamin (5–20 μM). After 1–2 days, media was collected and either diluted 1/10 before use in ELISA studies or let undiluted for other applications, such as Griess reaction.

Griess Reaction

NO production due to enhanced expression of iNOS in activated microglia was measured by a colorimetric method based on Griess reagent (Promega, Madison WI, no. G2930). The optical density (OD) was determined at 550 nm by an automated FLUOstar Optima reader (BMG Labtechnologies GmbH, Offenburg, Germany).

ELISA Analysis

Concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in the supernatant of primary microglial cells were quantified by standard ELISA techniques using the Duoset ELISA system (R&D, Minneapolis, MN) according to the manufacturer's instructions. Results represent the mean ± S.D. of three experiments, with each sample examined in triplicate within each experiment.

Cell Viability Assessment

Quantification of cell viability was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay at 0.5 mg/mL for 1 h. The reaction was terminated by removing the media and freezing the plate at −80° C for at least 1 h. Dimethyl sulfoxide (DMSO) solvent was added to each well for 1 h under shaking conditions at 37°C. The absorbance of each well was determined with an automated FLUOstar Optima reader (BMG Labtechnologies GmbH, Offenburg, Germany) at 570 nm with a reference filter at 630 nm. In addition, real time detection of cell proliferation was performed by measurements of cellular impedance by the xCELLigence system (Roche, Penzberg, Germany).

xCELLigence Impedance-Based System

xCELLigence system Real-Time Cell Analyzer RTCA-MP (Roche Diagnostics, Penzberg, Germany) allows tracking cellular impedance generated by cells, which after seeding, cover a micro-electrode network at the bottom of a culture dish. Cell resistance or impedance, depicted as cell index (CI) values and normalization were performed using the RTCA Software 1.2 (Roche Diagnostics). The normalization of cell index (NCIti) arbitrarily sets the CI values to 1 at the indicated time points. xCELLigence system provides cell characteristic kinetic traces based on cell number and morphology, and the degree to which they interact with the sensor surface. The cell resistance is increasing in a dependent way with both cell number and morphological alterations. In our study, cell impedance was used to monitor the real time kinetics of microglial morphology alterations (Diemert et al., 2012). Primary microglial cells were seeded at a density of 15,000 cells/well in 96-well E-plate (Roche Diagnostics). Prior to plating, background impedance was determined and always subtracted as blank value. Twenty-four hours after seeding, the cells were treated with different modulators of $K_{Ca}2$ channels in the presence or absence of LPS. At short time after each treatment, the CI drastically drops for 30–60 min due to the media change and temperature difference (Diemert et al., 2012), followed by a total recovery set in to values before media change or drug treatment.

Immunocytochemistry

Cells were fixed using paraformaldehyde (PFA) 4% for 20 min and permeabilized using Triton X 0.04% for 5 min. Unspecific binding was blocked by incubation with 3% of goat serum for 30 min. Incubation with primary antibody against F4/80, GFAP at a concentration of 1:100 was conducted overnight at 4°C. Secondary anti-rabbit antibodies coupled to Alexa FLUOR® 488, (Invitrogen, Karlsruhe, Germany) were incubated at room temperature. Nuclei were counterstained with DAPI. Images were acquired using a confocal laser scanning microscope (Axiovert 200, Carl Zeiss, Jena, Germany) equipped with an UV and an argon laser delivering light at 364 nm and 488 nm, respectively. Light was collected through a 63 × 1.4 NA or 100 × 1.3 NA oil immersion objectives.

Protein Analysis

Primary microglia were lysed in 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X, pH 7.4, complete mini protease inhibitor cocktail tablet, and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich). The membranes were incubated overnight with primary antibodies (1:3,000 and 1:1,000; rabbit anti-$K_{Ca}2.2$ and -$K_{Ca}2.3$ channel (Sailer et al., 2004) at 4°C and afterwards with peroxidase-conjugated secondary antibodies (1:5,000).

Transfection

Cellular loading of peptides was performed with cationic lipid mixture Pro-Ject™ Protein Transfection Reagent kit according to the manufacturer's instructions (Pierce Prod no. 89850). Prediction of coiled-coil domains of the C-terminus of $K_{Ca}2$ channels by Coils Version 2.2 program (http://www.ch.embnet.org) and the characterization of peptide specificity for $K_{Ca}2$ channels was performed by Tuteja et al. (2010). Microglial cells were transfected with 50 μM peptides in 96-well plates (15,000–17,000 cells/well) for 48 h. The evaluation of the impact of these peptides was carried out by microscope and by the impedance measurements (xCELLigence, Roche, Penzberg).

RT-PCR

Total RNA was extracted using the NucleoSpin RNA II kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. RT reac-

tions were conducted using SuperScript® III One-Step RT-PCR System (Invitrogen) in a Lab Cycler, Sensoquest (SensoQuest Biomedizinische Elektronik GmbH, Göttingen, Germany). Following primers synthesized by Eurofins MWG Operon (Ebersberg, Germany) were used: *KCNN1*/SK1/K_{Ca}2.1 (240 bp) 5′-CTGTGGGAAGGGCGT GTGTCTG-3′; 5′-CCGAACCCGGCTTTGGTCTGG-3′; *KCNN2*/SK2/K_{Ca}2.2 (220 bp) 5′-GTGCTCTTGGTTT TAG-TATCTCG-3′; 5′-CAACCTGCACCCATTATTCC-3′; *KCN N3*/SK3/K_{Ca}2.3 (400 bp) 5′-GCCAACAAGCGGAAAAA CCAAAAC-3′; 5′-CCAGGCGTGCCGTCCAGAAGAAC-3′; *KCNN4*/SK4/IK/K_{Ca}3.1 (410 bp) 5′-GCATCGGGGCGCT-CAACCA-3′; 5′-AGCGGCCG ACTCCTTCATCTCTTT-3′. cDNAse digestion and RT-PCR without RT in the reaction mixture were used as negative controls for genomic DNA contamination. The following amplification of cDNA by PCR using specific primers were carried at the following steps: (1) denaturing at 95°C for 4 min; (2) 94°C for 30 sec; (3) T_m (annealing temperature) for 30 sec, depending on the *KCNN*/SK/K_{Ca}2 isoform of interest; (4) extension at 72°C for 30 sec; with 30 cycles from Steps 2 to 4. The final extension step was set to 72°C for 5 min. The T_m for *KCNN1*/SK1/K_{Ca}2.1 was 63°C; 57.3°C for *KCNN2*/SK2/ K_{Ca}2.2; 61°C for *KCNN3*/SK3/K_{Ca}2.3 (Dolga et al., 2011).

Calcium Measurements

To study the effects of LPS on $[Ca^{2+}]_i$, we used a ratiometric calcium dye, Fura-2AM. Sub-cultured primary microglia grown on Ibidi μ-slides (Ibidi GmbH, Martinsried, Germany) at a density of 15,000–17,000 cells/well, for 2–3 days was incubated with 2 μM FURA-2 AM for 30 min at 37°C in HEPES-ringer buffer. Fluorescence intensities from single cells excited at the two wavelengths (F340 and F380) were recorded separately and combined (fluorescence ratio: r = F340/F380) after background subtraction (fluorescence of a cell-free area). Measurements were performed on 20–30 cells per well (with at least three wells per condition). For assessment of calcium levels, measurements were performed on random spots of Ibidi μ-slides without the treatment history knowledge and the data were pooled independently of microglial morphological shape. Experiments were repeated at least three times using primary microglia cultures from independent preparations.

Statistical Analysis

All data are given as means ± S.D. For statistical comparisons between two groups, Student's t-test was used assuming a normal distribution of the respective data (analyzed with Shapiro–Wilk test) and U-test Mann–Whitney (for ELISA and NO measurements from six different wells and repeated for three times with independent primary microglial culture preparations). Multiple comparisons were performed by ANOVA followed by Scheffé's and Bonferoni *post hoc* test (for MTT assays and calcium measurements, six different wells, repeated at least three times with independent primary microglial culture preparations). Calculations were made with the Winstat standard statistical software package (Robert Fitch Software, Bad Krozingen, Germany). A statistically significant difference was assumed at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

RESULTS

Activation of *KCNN*/SK/K_{Ca}2 Channels Reverses LPS-Induced Microglial Activation

To study the impact of *KCNN*/SK/K_{Ca}2 channels on the activation of microglial cells, we first established LPS-induced microglial activation kinetics using real-time impedance measurements (Diemert et al., 2012). These measurements provide information on microglial morphological changes, which are continuously monitored for the whole period of LPS exposure. LPS is commonly used to induce activation of microglial-macrophage cells and in studies of bacterial infections. Impedance alterations were displayed as CI and suggested that LPS induced a continuous concentration-response increase in microglial proliferation (Fig. 1A), which were detected within 90 min following LPS exposure. This increase in CI persisted during the following 24 h (Fig. 1B). The impedance measurements were paralleled by immunofluorescence studies with a microglial marker (F4/80 antibody) (Fig. 1C). We found that LPS induced marked alterations of microglial structural features in a concentration-dependent manner as detected by both xCELLigence measurements and F4/80 immunostainings. Based on these results and on clinically relevant concentration ranges (Hanisch et al., 2001), we used 200 ng/mL LPS in further experiments of the study.

To dissect the role of K_{Ca}2 channels in activated microglia following the LPS challenge, we used pharmacological modulators of *KCNN*/SK/K_{Ca}2 channels and tested their influence on microglial morphological changes and proliferation. First, we established the expression levels of *KCNN*/SK/K_{Ca}2 channels by RT-PCR analysis. In primary microglia cells, mRNA analysis by RT-PCR revealed expression of all *KCNN1*/SK1/ K_{Ca}2.1, *KCNN*/SK2/K_{Ca}2.2, and *KCNN*/SK3/K_{Ca}2.3 channel subtypes (Fig. 1D). As a positive control, we documented the expression of K_{Ca}3.1 channel subtype that is known to be highly expressed in microglia (Kaushal et al., 2007). Next, we assessed the appropriate concentration and time kinetics of the positive *KCNN2*/3/ K_{Ca}2.2/K_{Ca}2.3 channel modulator CyPPA in primary microglia (Fig. 1E,F). Western blot analysis showed that CyPPA alone is not altering the *KCNN*/SK2/K_{Ca}2.2 or *KCNN*/SK3/K_{Ca}2.3 channel expression (Fig. 1E). MTT analysis showed that at low concentrations of 10–25 μM, CyPPA did not affect cellular survival even after 48 h. However, at concentrations higher than 75 μM CyPPA considerably reduced cellular viability after 24 h (Fig. 1F). Based on these results, we used concentrations below 75 μM CyPPA in our further studies.

Co-treatment of microglia with LPS and CyPPA promoted a strong reduction of LPS-induced CI increase in a concentration-dependent manner. However, CyPPA to-

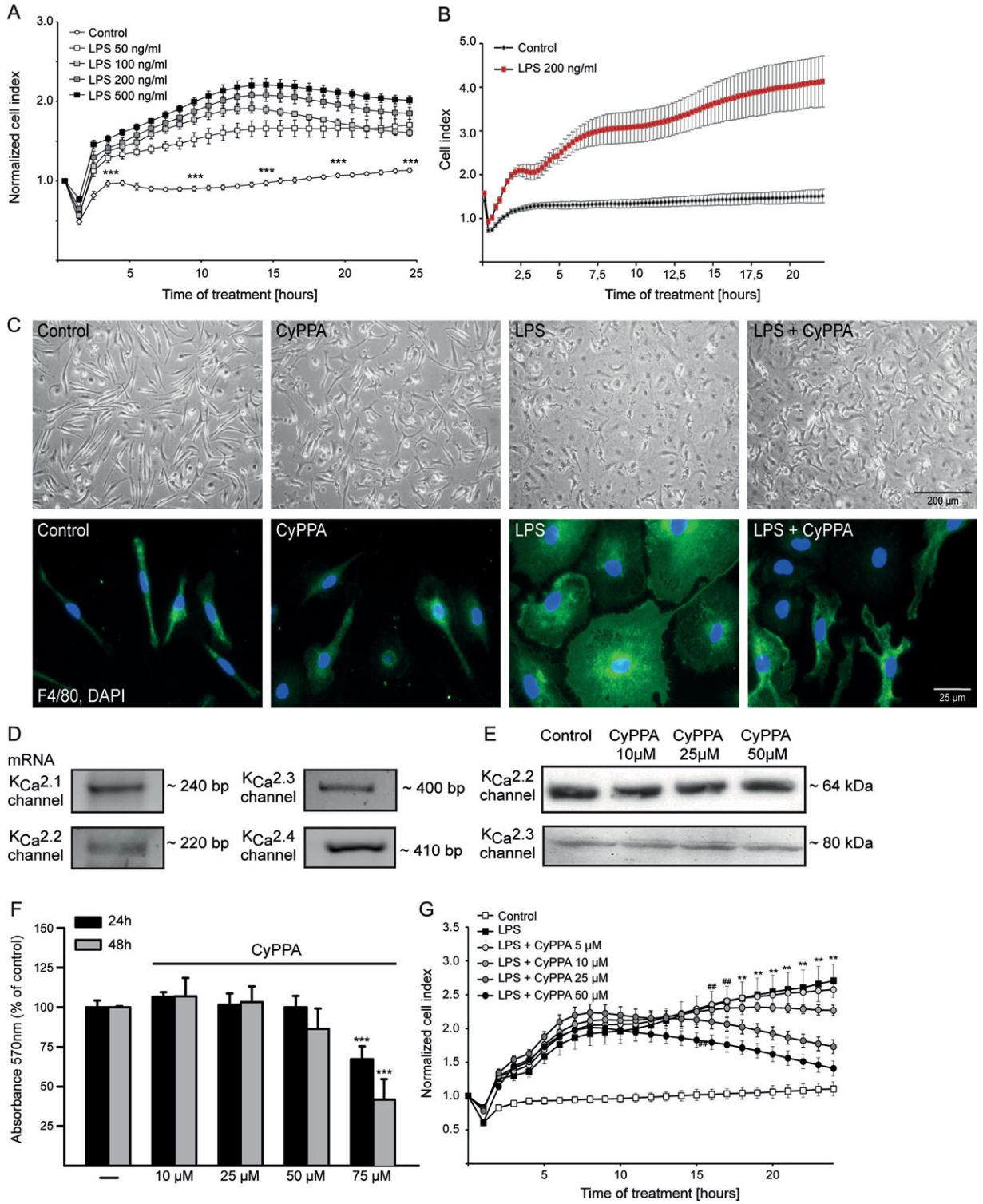

Fig. 1. LPS induces primary microglial activation. **A**: Microglial cells were seeded in 96-well E-plates at a density of 15,000 cells/well and monitored with a real-time impedance-based xCELLigence system. After 24 h, cells were challenged with different concentrations of LPS, ranging from 50 to 500 ng/mL, as indicated. The time point of LPS treatment or media change is marked as "0 h" in the graph. **B**: Representative LPS kinetic curve using xCELLigence system ($n = 6$ wells). The time point of LPS treatment or media change is marked as "0 h" in the graph. **C**: Morphometric alterations of activated microglia were visualized by bright-field microscope and by immunostaining with F4/80 antibody. **D**: mRNA analysis of *KCNN*/SK/K_{Ca}2 channel subtypes. **E**: Western blot analysis of *KCNN2*/SK2/K_{Ca}2.2 and *KCNN3*/SK3/K_{Ca}2.3 channels in microglial cells treated in the presence or absence of different concentrations of CyPPA, ranging from 10 to 50 µM. **F**: MTT analysis of microglial cells treated with different concentrations of CyPPA for 24 and 48 h. Results are given as mean values ± S.D. (***$P < 0.001$ versus non-treated microglia, ANOVA, Scheffé's test, $n = 18$ wells, repeated three times with independent primary microglia preparations). **G**: xCELLigence analysis of microglial cells treated with 200 ng/mL LPS, in the presence or absence of different concentrations of CyPPA (5–50 µM), as indicated. The time point of treatment is marked as "0 h" in the graph (**$P < 0.01$ versus CyPPA-treated microglia (25 µM), ##$P < 0.01$ versus CyPPA-treated (50 µM) microglia, ANOVA, Scheffé's test, $n = 6$ wells, experiment repeated at least three times with independent primary microglia preparations). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

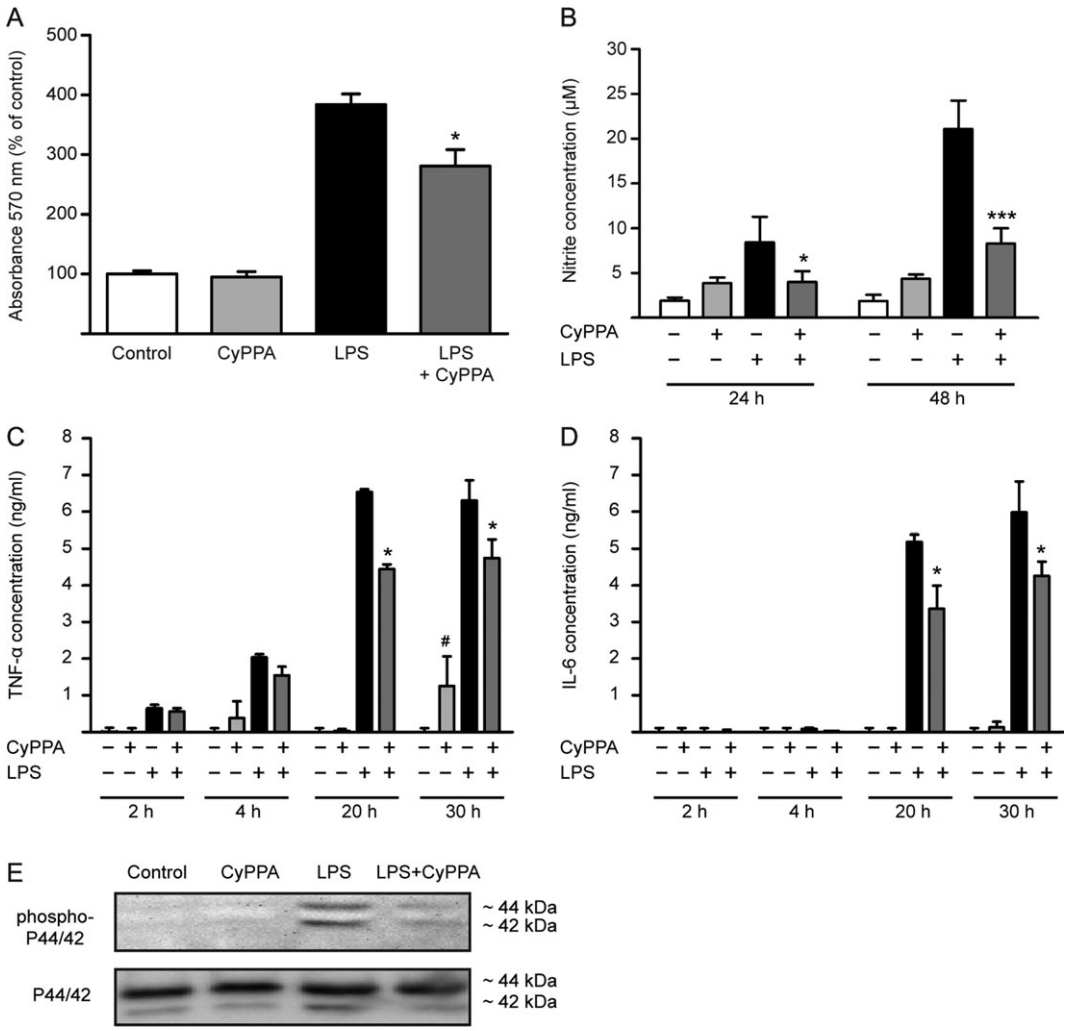

Fig. 2. CyPPA prevents cytokine release. **A**: MTT analysis of microglial cells treated with CyPPA (25 μM) for 24 h in the presence of LPS (200 ng/mL). Results shown represent mean ± S.D. (*$P < 0.05$ versus LPS-treated microglia, ANOVA, Scheffé's test, $n = 6$ wells, experiment repeated at least three times with independent primary microglia preparations). **B**: NO production of microglial cells treated with 200 ng/mL LPS in the presence and absence of CyPPA (25 μM). The effects of CyPPA (25 μM) on cytokine production, TNF-α (**C**) and IL-6 (**D**) in LPS (200 ng/mL)-activated microglia for 2–30 h. Results represent mean S.D. (*$P < 0.05$; ***$P < 0.001$ versus LPS-treated microglia; #$P < 0.05$ versus non-treated cells, *U*-test Mann–Whitney, $n = 3$). **E**: Western blot analysis of phosphorylated and non-phosphorylated p44/p42 MAPK in microglial cells treated in the presence or absence of CyPPA (25 μM) and LPS (200 ng/mL).

gether with LPS did not reduce the first increase in the cell index, but produced a decline in CI values following 8–10 h after CyPPA application (Fig. 1G). Using the xCELLigence system, we determined the IC_{50} of 19 μM for CyPPA at 24 h after LPS application. Immunofluorescence analysis of specific microglial markers, such as F4/80 proteins, showed that LPS enlarged microglial cell bodies, which was prevented by CyPPA (Fig. 1C). To exclude toxic effects of CyPPA in the presence of LPS, we analyzed metabolic activity using an MTT assay. CyPPA exposure for 24 h reduced LPS-dependent microglial activation, but showed no alterations in the MTT assay when compared with non-treated microglia (Fig. 2A). Thus, the CyPPA-induced decline in the CI determined by impedance measurements was unlikely related to a reduced viability of microglial cells 24 h after the LPS challenge.

To further evaluate the impact of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels on microglial activation processes, we investigated NO production and cytokine release in response to positive modulation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels. CyPPA (25 μM) significantly reduced the LPS-mediated NO production in microglial cells at 24 and 48 h (Fig. 2B). Notably, CyPPA alone slightly increased NO release under control conditions but still blocked the burst of NO production induced by LPS (Fig. 2B).

Similarly, activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels affected LPS-induced cytokine release in microglia. Under control conditions, the cytokines were not detectable in the supernatant of microglial cells. Activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels did not induce cytokine production at earlier time points (Fig. 2C,D). However, CyPPA increased TNF-α

release after 30 h treatment without altering the production of IL-6 cytokine. LPS caused an extracellular accumulation of the cytokines TNF-α as early as 4 h, which was partially reduced by activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels (Fig. 2C). This reduction of LPS-dependent cytokine TNF-α and IL-6 release was even more pronounced after 20 and 30 h, suggesting a strong inhibitory effect on inflammatory responses in microglia by *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channel activation (Fig. 2C,D).

To further determine the effects of CyPPA on LPS-dependent microglial activation and proliferation, we assessed the levels of phosphorylated p44/42 mitogen-activated protein kinase (MAPK) protein, a well-established marker for cell proliferation and microglial activation (Koistinaho and Koistinaho, 2002; Liva et al., 1999). Western blot analysis showed that LPS promoted p44/42 MAPK phosphorylation, effect prevented by activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels with CyPPA (Fig. 2E).

Pretreatment with *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 Channel Activators Prevents Microglial Activation

To investigate the contribution of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels in microglial activation pathways, we pretreated primary microglia with CyPPA for 24 h prior to LPS application. Such activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels strongly prevented the morphological alterations mediated by LPS, as detected by impedance measurements (Fig. 3A).

In addition, LPS-induced NO production as a result of microglial activation was drastically reduced by pretreatment with CyPPA (Fig. 3B). The analysis of cytokine release revealed that TNF-α and IL-6 production were also attenuated when microglia were pretreated for 24 h with the *KCNN2/3*/ K_{Ca}2.2/K_{Ca}2.3 channel activator (Fig. 3C).

To further evaluate the effect of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels on microglial activation, we performed experiments with CyPPA at different time points after the initiation of LPS-induced microglial activation. Microglia received CyPPA (25 μM) 2, 4, 6, and 24 h following the LPS challenge. xCELLigence impedance analysis showed that CyPPA was able to block microglial proliferation in the first hours of LPS treatment (Fig. 3D). Further, CyPPA was able to reduce the CI even when applied only 24 h after the onset of the LPS challenge, suggesting that *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels also affected ongoing microglial activation pathways.

KCNN/SK/K_{Ca}2 Channel Activator CyPPA Controls Microglial Activation Through Effects on Intracellular Calcium Levels

Next, we addressed the question, whether LPS was still capable of inducing microglial activation in the absence of extracellular Ca^{2+}. We compared LPS effects on microglial cells in calcium-containing medium with effects obtained in calcium-free medium. Impedance measurements showed that activation of microglial cells was drastically reduced in the absence of extracellular calcium (Fig. 4A,B). These experiments were also paralleled by MTT proliferation assays, which confirmed that activation of microglia was significantly diminished in the absence of extracellular calcium (Fig. 4C). Further, the cells were challenged with LPS in the presence of the calcium chelator EDTA to substantiate the role of extracellular calcium in LPS-induced microglial activation. Chelating extracellular calcium by EDTA attenuated microglial activation in a concentration- and time-dependent manner (Fig. 4D). It is interesting to note that EDTA alone did not influence cell proliferation when compared with controls, while a combination of LPS and EDTA reduced cell proliferation in the microglial cultures compared with LPS treatment alone (Fig. 4E). These findings indicate that microglial activation depends on extracellular calcium.

Chronic elevation of basal $[Ca^{2+}]_i$ in response to LPS exposure is a central element in microglial activation processes (Hoffmann et al., 2003). To further study the proposed role of increased $[Ca^{2+}]_i$, levels for the activation of microglial cells, we performed time kinetics using a ratiometric calcium indicator, Fura-2 AM. CyPPA alone did not cause any significant alterations of $[Ca^{2+}]_i$ compared with control microglia cultures (Fig. 4F). Thus, activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels did not affect calcium signaling *per se* under control conditions. To obtain further insight in microglial calcium signaling, we monitored $[Ca^{2+}]_i$, for a period of 30 h after the onset of LPS challenge. Alteration in calcium levels were analyzed in microglial cells treated for 2, 4, 8, 20, and 30 h with LPS. Interestingly, using this approach, LPS induced an increase in $[Ca^{2+}]_i$, levels upon 2 h of LPS application, followed by a reduction in $[Ca^{2+}]_i$ signals after 4 h of LPS challenge, followed by higher values than non-treated microglia, after 20 h of LPS application (Fig. 4G). This striking reduction in $[Ca^{2+}]_i$ correlated well with the previous report on the LPS-dependent effects on $[Ca^{2+}]_i$ kinetics (Beck et al., 2008). Microglial cells challenged with LPS for at least 30 h in the presence of CyPPA, however, did not show the previously observed LPS-induced elevation of $[Ca^{2+}]_i$ (Fig. 4H). Interestingly, CyPPA attenuated LPS-mediated calcium increases already after 2 h of LPS challenge. This result suggests that activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels could prevent LPS-induced alterations in microglial $[Ca^{2+}]_i$, (Fig. 4H).

KCNN3/SK3/K_{Ca}2.3 Channels but Not *KCNN2*/SK2/K_{Ca}2.2 Channels Prevent Microglial Activation by LPS

To probe the proposed role of *KCNN*/SK/K_{Ca}2 channels in microglial activation, we treated microglial cells with apamin, a non-selective *KCNN*/SK/K_{Ca}2 channel subtype pore blocker. Microglial proliferation was not affected by apamin alone, while the CyPPA-dependent decrease of cell index in activated microglia was clearly blocked by

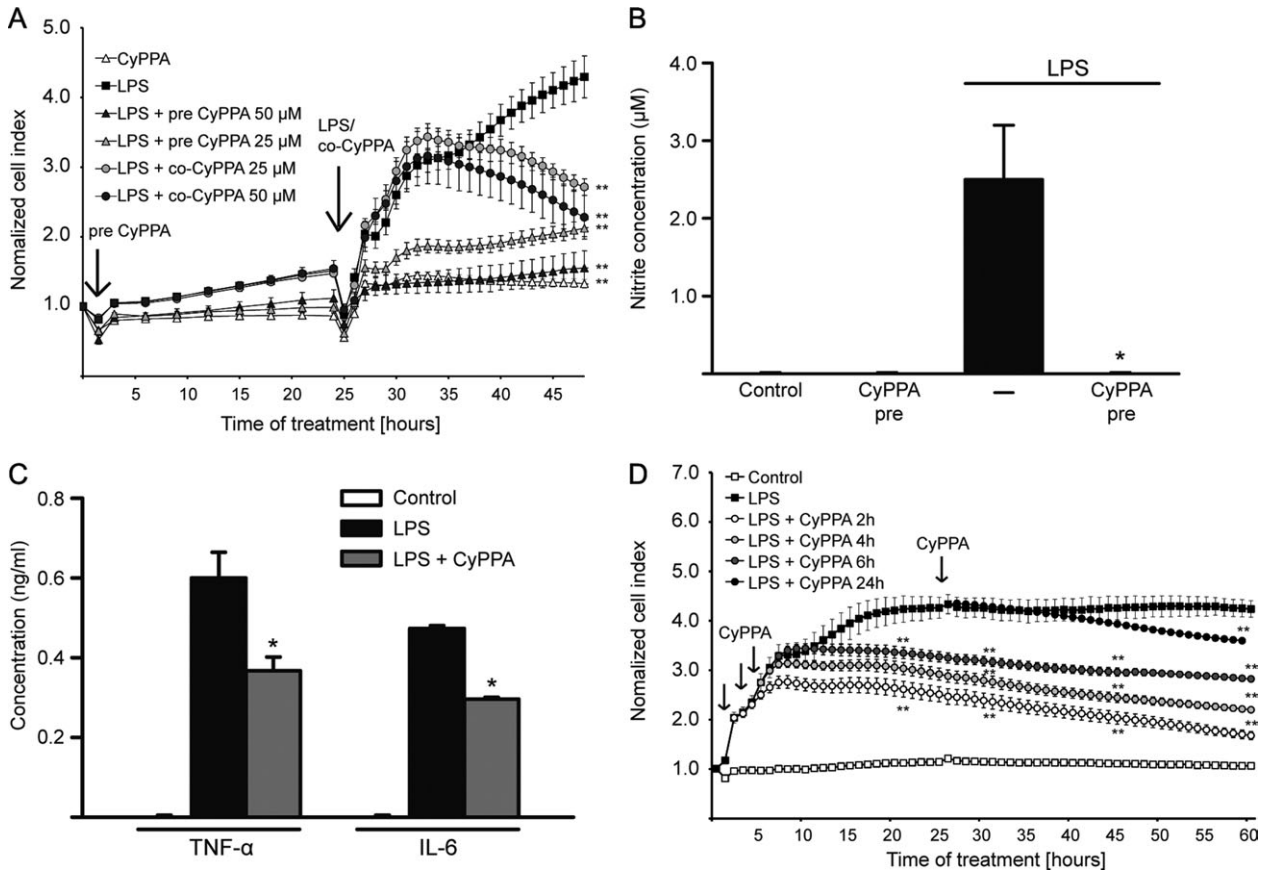

Fig. 3. Activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels prevents microglial activation. **A**: Microglial cells were seeded in 96-well E-plates with a density of 15,000 cells/well and monitored with a real-time xCELLigence impedance-based system. Some microglial cultures were pre-treated with CyPPA (25 and 50 µM) for 24 h (indicated as "LPS+pre CyPPA"). Afterwards, cells with or without CyPPA pre-incubation were challenged with LPS (200 ng/mL), as indicated by an arrow on the kinetic curve. To demonstrate that CyPPA is able to reduce LPS-induced cell index increase, some microglia were co-treated with LPS and CyPPA (indicated as "LPS+co-CyPPA"). The time point of CyPPA treatment initiation or media change is marked as "0 h" and of LPS challenge as "24 h" (n = 6 wells, experiment repeated at least three times with independent primary microglia cultures, $^{**}P < 0.01$ versus LPS-treated microglia, ANOVA, Scheffé's test). **B**: NO production of microglial cells pre-treated with CyPPA (25 µM) in the presence and absence of LPS (200 ng/mL) ($^{*}P < 0.05$ versus LPS-treated microglia, *U*-test Mann–Whitney, n = 3, experiment repeated three times with independent primary microglia cultures). **C**: Cytokine production in microglial cells pre-treated with CyPPA (25 µM, 24 h) and followed by LPS challenge (200 ng/mL, 8 h). ($^{*}P < 0.05$ versus LPS-treated microglia, *U*-test Mann–Whitney, n = 3, experiment repeated at least three times with independent primary microglia cultures). **D**: Microglial cells were challenged with LPS (200 ng/mL) followed by CyPPA treatment 2, 4, 6, and 24 h post-LPS application (as indicated by black arrows on the graph). Microglial cells were monitored with a real-time xCELLigence impedance-based system for 60 h. The time point of LPS treatment initiation or media change is marked as "0 h" in the graph (n = 4–6 different wells, experiment repeated at least three times with independent primary microglia cultures, $^{**}P < 0.01$ versus LPS-treated microglia, ANOVA, Scheffé's test).

apamin (Fig. 5A). To further differentiate the contribution of the individual *KCNN*/SK/K_{Ca}2 channel subtypes in LPS-induced microglial activation, we used specific inhibitory peptides for each *KCNN*/SK/K_{Ca}2 channel subtype expressed in microglia, i.e., *KCNN1*/SK1/K_{Ca}2.1, *KCNN2*/SK2/K_{Ca}2.2, and *KCNN3*/SK3/K_{Ca}2.3 channels (Tuteja et al., 2010). Since these inhibitory peptides bind to the intracellular C-terminus, we used the Pro-Ject™ transfection reagent to ensure intracellular uptake of the respective peptide inhibitors. The transfection efficacy of the inhibitory peptides was assessed with rhodamine-labeled control peptides which showed a transfection rate of greater than 90 % (Fig. 5E).

Application of specific inhibitory peptides for *KCNN1*/SK1/K_{Ca}2.1 and *KCNN2*/SK2/K_{Ca}2.2 channels did not influence CyPPA effect on LPS-induced microglial proliferation. In sharp contrast, the specific inhibitory peptides for *KCNN3*/SK3/K_{Ca}2.3 channels delayed the CyPPA effect for at least 10–15 h, suggesting that CyPPA blocked LPS-induced activation of microglia through enhanced activity of *KCNN3*/SK3/K_{Ca}2.3 channels (Fig. 5B–D).

LPS-Induced NO and Cytokine Release by Microglial Cells are Altered by Modulation of *KCNN3*/SK3/K_{Ca}2.3 Channels

Next, we determined the effect of the *KCNN*/SK/K_{Ca}2 channel blocker apamin and the specific inhibitory peptides on NO production, as well as the release of

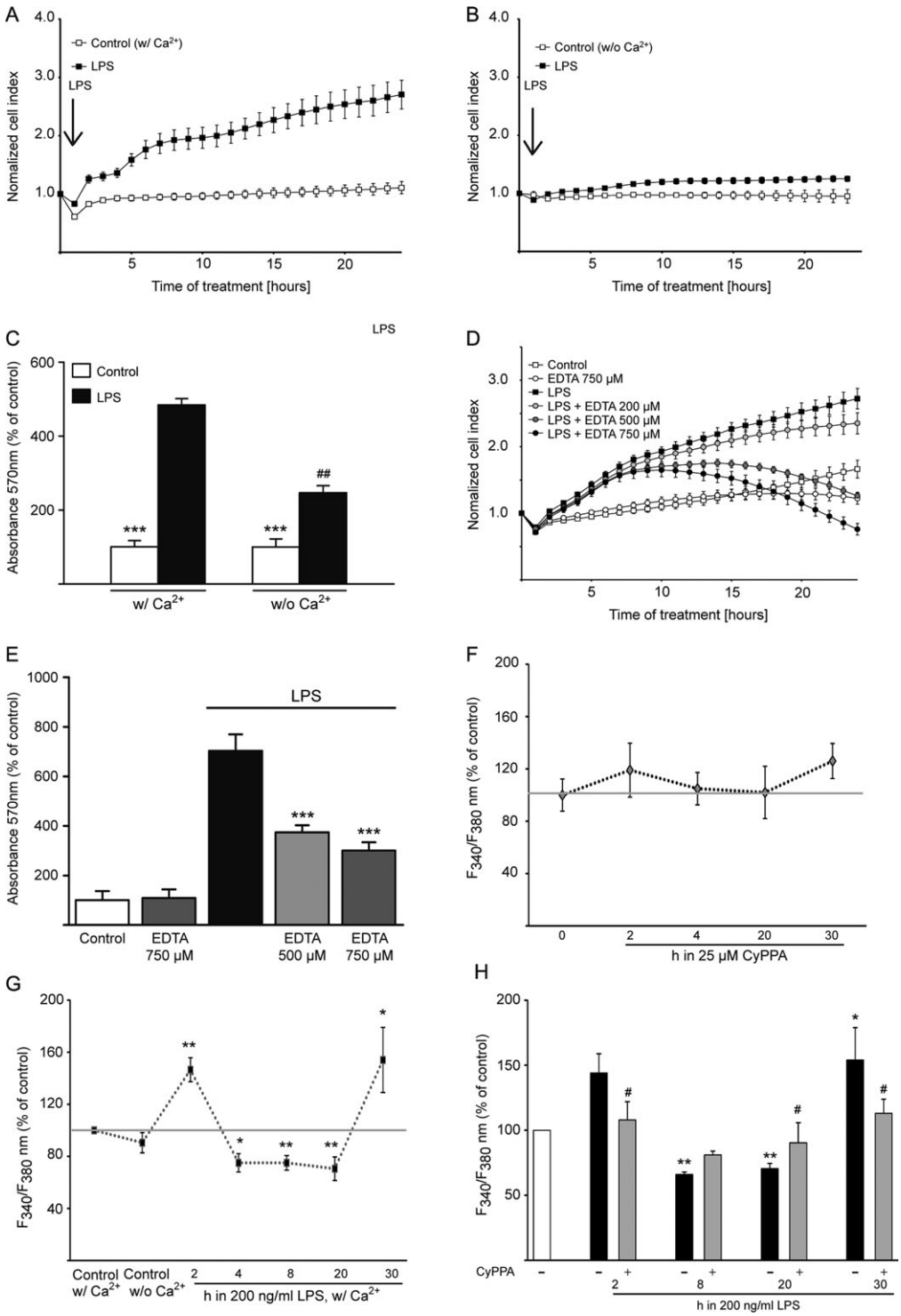

Fig. 4. Extracellular calcium is a prerequisite for microglial activation**.** Morphological alterations of microglial cells were detected by real-time impedance-based system. **A**: Microglia was challenged with 200 ng/mL LPS in the presence of extracellular calcium or (**B**) in calcium-free medium. The time point of treatment is marked as "0 h" in the graph (n = 6 different wells, experiment repeated three times with independent primary microglia cultures). **C**: MTT analysis of microglial cells challenged with LPS (200 ng/mL) in calcium-containing medium as well as in calcium-free medium (***$P < 0.001$ versus LPS-treated microglia, ##$P < 0.01$ versus LPS-treated cells in calcium-containing medium, ANOVA, Scheffé's test, n = 6 different wells, experiment repeated three times with independent primary microglia cultures). **D**: xCELLigence measurements of LPS-activated cells co-treated with different concentrations of EDTA, ranging from 200 to 750 µM, as indicated. **E**: MTT analysis of microglia activated by 200 ng/mL LPS in the presence of the extracellular calcium chelator, EDTA (500 and 750 µM). Results represent mean ± S.D. (***$P < 0.001$ versus LPS-treated microglia, ANOVA, Scheffé's test, n = 6, experiment repeated three times with independent primary microglia cultures). **F**–**H**: Calcium measurements were performed with Fura-2AM calcium sensor. Basic intracellular Ca^{2+} changes (F340/F380) during the first 30 h (F, n = 3 independent experiments from different primary microglial preparations, with 30–40 cells measured per condition) after incubation with 25 µM CyPPA (F) or 200 ng/mL LPS (**G**). (n = 30–40 cells, **$P < 0.01$ versus untreated microglia, #$P < 0.05$ versus LPS-treated microglia, ANOVA, Scheffé's test, n = 3 independent experiments). **H**: Microglia cells were challenged with 200 ng/mL LPS for 2–30 h. Some cells were treated with CyPPA (25 µM) and the $[Ca^{2+}]_i$ was measured with Fura-2AM calcium sensor. Results represent mean ± S.D. (***$P < 0.001$ versus LPS-treated microglia, ANOVA, Scheffé's test, n = 3).

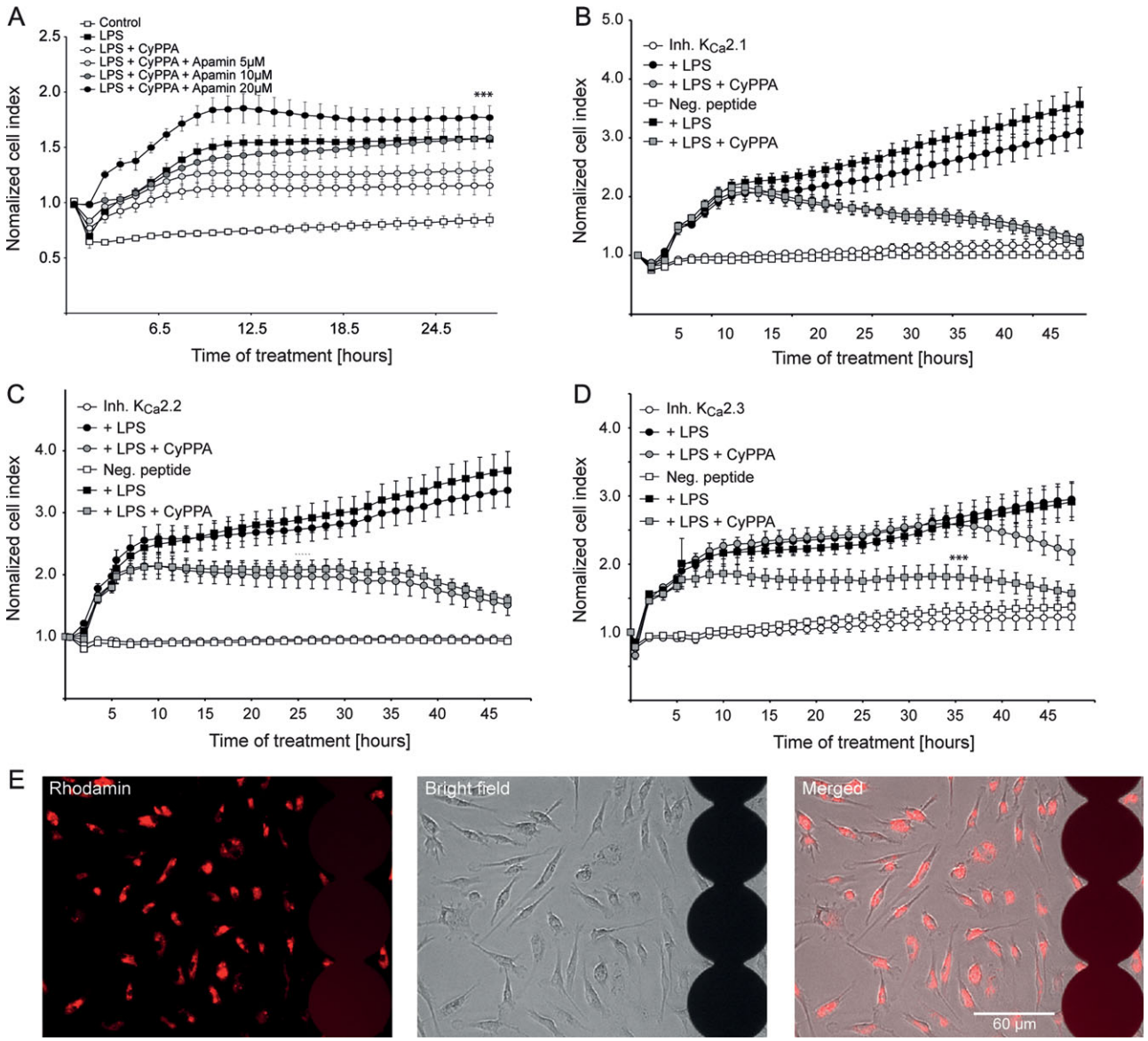

Fig. 5. Prevention of microglial activation is dependent on *KCNN3*/SK3/KCa2.3 channels. **A**: Microglial cells were seeded in 96-well E-plates with a density of 15,000 cells/well and monitored with a real-time xCELLigence impedance-based system. Microglial cells were challenged with LPS (200 ng/mL) and co-treated with CyPPA (25 μM) in the presence and absence of different concentrations of apamin. The time point of pre-treatment is marked as "0 h" in the graph (n = 6 wells, experiment repeated three times with independent primary microglia cultures, $^{***}P < 0.001$ versus CyPPA-treated microglia, ANOVA, Scheffé's test). **B**: xCELLigence measurements of microglial cells transfected with inhibitory peptides specific for (B) *KCNN1*/SK1/K_{Ca}2.1, (**C**) *KCNN2*/SK2/K_{Ca}2.2 or (**D**) *KCNN3*/SK3/K_{Ca}2.3 channels. Cells were treated with 200 ng/mL LPS in the presence or absence of 25 μM CyPPA. The time point of pre-treatment is marked as "0 h" in the graph (n = 6, $^{***}P < 0.001$ versus LPS+CyPPA-treated microglia transfected with K_{Ca}2.3 inhibitory peptides, ANOVA, Scheffé's test). **E**: The transfection efficacy of the ProJect™ transfection kit was assessed with rhodamine-labeled peptides (red fluorescence). The photomicrographs show the transfected cells growing on the electrode layer (black circles) of E-plates that enable cellular impedance measurements. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cytokines, i.e., characteristic features of microglial inflammatory responses. Interestingly, apamin reversed the inhibitory effects of CyPPA on LPS-induced cytokine releases (Fig. 6A,B). Since apamin is a general *KCNN*/SK/K_{Ca}2 channel blocker, we further investigated NO and cytokine release in the presence of specific inhibitory *KCNN*/SK/K_{Ca}2 channel peptides, in order to distinguish particular contributions of the individual K_{Ca}2 channel subtypes. The peptides targeting the *KCNN3*/SK3/K_{Ca}2.3 channel abolished the CyPPA-mediated inhibition of LPS-induced NO production (Fig. 6C). Interestingly, CyPPA reduced TNF-α release in activated microglia even in the presence of K_{Ca}2.3 channel inhibitory peptides (Fig. 6D). These data show that the TNF-α release activities are not modulated by *KCNN3*/SK3/K_{Ca}2.3 channels. In contrast, IL-6 release was not reduced by CyPPA in the presence of *KCNN3*/SK3/K_{Ca}2.3 inhibitory peptides when compared with the cor-

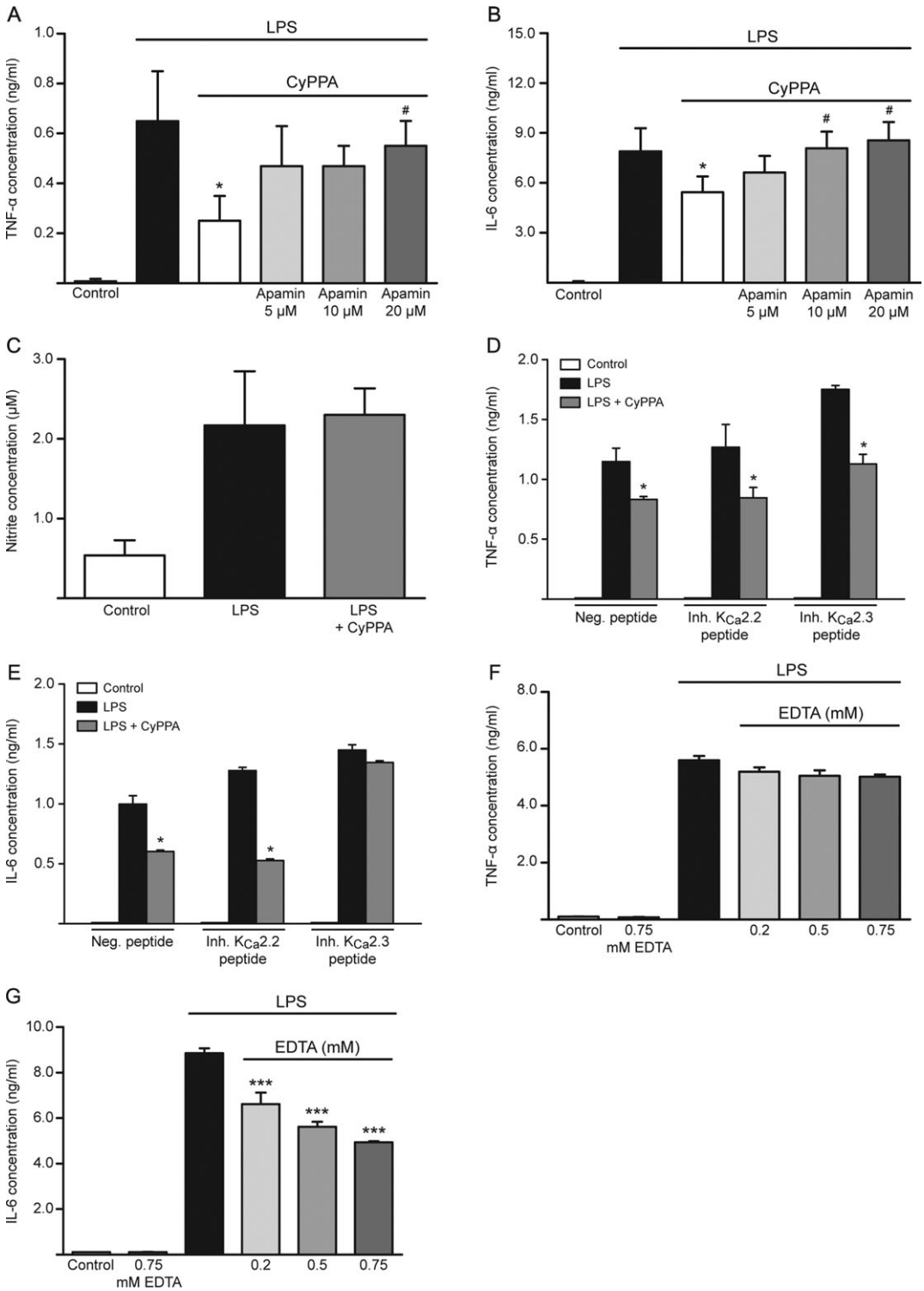

Fig. 6. KCa2.3 regulates microglial activation pathways. Cytokine production, TNF-α (**A**) and IL-6 (**B**) in microglial cells co-treated with LPS (200 ng/mL) and CyPPA (25 μM) in the presence and absence of different concentrations of apamin. Results represent mean ± S.D. ($^{*}P < 0.05$ versus LPS-treated microglia, $^{\#}P < 0.05$ versus CyPPA-treated microglia, *U*-test Mann–Whitney, $n = 3$, experiment repeated three times with independent primary microglia cultures). **C**: NO release in microglial cells transfected with inhibitory peptides for *KCNN3*/SK3/K_{Ca}2.3 channels. Results shown represent mean ± S.D. ($n = 3$, experiment repeated three times with independent primary microglia cultures). **D**: TNF-α and (**E**) IL-6 production in microglial cells transfected with inhibitory peptides for *KCNN2*/SK2/K_{Ca}2.2 and *KCNN3*/SK3/K_{Ca}2.3 channels and challenged with LPS (200 ng/mL) in the presence of CyPPA (25 μM). Results are presented as mean values ± S.D. ($^{*}P < 0.05$ versus LPS-treated microglia, *U*-test Mann–Whitney, $n = 3$, experiment repeated three times with independent primary microglia cultures). **F**: TNF-α and (**G**) IL-6 production in microglial cells and co-treated with different concentrations of EDTA, as indicated. Results are shown as mean values ± S.D. ($^{***}P < 0.001$ versus LPS-treated microglia, *U*-test Mann–Whitney, $n = 6$, experiment repeated three times with independent primary microglia cultures).

responding LPS-activated microglia (Fig. 6E). These results strongly suggested that IL-6 release was regulated by *KCNN3*/SK3/K_{Ca}2.3 channel subtypes.

To determine the impact of calcium signaling on cytokine release, we analyzed the cytokine production in response to microglial activation when extracellular calcium was chelated with various concentrations of EDTA. It was previously shown that reducing $[Ca^{2+}]_i$ with BAPTA-AM strongly attenuated cytokine release in activated microglia (Hoffmann et al., 2003). However, treatment with ionomycin was demonstrated to enhance $[Ca^{2+}]_i$ without any cytokine production, suggesting that increases in $[Ca^{2+}]_i$ are permissive but cannot trigger the cytokine production and release by itself (Hoffmann et al., 2003). In our studies, chelating extracellular calcium in LPS-stimulated microglia did not alter TNF-α release (Fig. 6F). Interestingly, in sharp contrast to the results obtained with TNF-α measurements, the chelation of extracellular calcium with EDTA strongly attenuated the IL-6 release in a dose-dependent manner (Fig. 6G). These experiments suggest that *KCNN3*/SK3/K_{Ca}2.3 activation attenuated IL-6 release because of reduced Ca^{2+} influx, whereas TNF-α release is regulated in a calcium-independent manner.

DISCUSSION

The results of the present study demonstrate that in primary microglial cells, *KCNN3*/SK3/K_{Ca}2.3 channel activation attenuates LPS-induced increases in $[Ca^{2+}]_i$, morphological transformation to macrophages, cell proliferation, and IL-6 production. Persistent elevation of $[Ca^{2+}]_i$ was previously reported in mouse microglia after 24 h of LPS treatment (Hoffmann et al., 2003). There is an ongoing debate on the mechanisms underlying receptor-mediated microglial activation and calcium homeostasis. Several hypotheses imply that an elevation of $[Ca^{2+}]_i$ in microglial cells results from altered efficiencies of Ca^{2+} extrusion pumps and insufficient intracellular Ca^{2+} buffering systems. Several ion channels have been reported to act in concert to shape the calcium signals in microglial cells (Beck et al., 2008; Launay et al., 2004). Sub-cultured microglial cells express inward rectifying potassium currents (I_{IRK}), voltage-gated, and delayed rectifying potassium currents (I_{DRK}), including Kv1.3 and Kv1.5 channels (Beck et al., 2008; Boucsein et al., 2000; Kettenmann et al., 1990). In addition, transient receptor potential cation channel subfamily M member 4 (TRPM4), a Ca^{2+}-activated Ca^{2+} current (I_{CRAC}) was shown to regulate Ca^{2+} oscillations by membrane potential changes (Beck et al., 2008; Launay et al., 2004). During several days of sub-culturing microglia, the percentage of cells that exhibit potassium I_{IRK} current increased, and the percentage of cells with prominent potassium I_{DRK} current drastically declined (Beck et al., 2008). Following 24 h LPS challenge, 14% of cells revealed I_{IRK} current and 3% I_{DRK} current, suggesting that I_{IRK} current is essential for LPS-dependent Ca^{2+} alterations. In the present study, we reveal a significant contribution of *KCNN*/SK/K_{Ca}2 channels in regulating the calcium homeostasis of microglial cells. In fact, the *KCNN3*/SK3/K_{Ca}2.3 subtype of these channels apparently controls increases in $[Ca^{2+}]_{i,}$, microglial activation, and inflammatory cytokine release.

However, the association between microglial morphological changes, alterations of intracellular $[Ca^{2+}]$ and cytokine release has not been fully elucidated so far. Most of the previous reports from studies in sub-cultured microglia showed the effects of LPS on morphological parameters and on Ca^{2+} signals after 24 h, without showing data of the time window before the full microglial activation. Our impedance measurements revealed that LPS altered microglial morphological shape immediately following its application. The LPS-induced microglial impedance kinetic demonstrated that there are three phases of microglial activation, first phase includes a sharp, fast increase of cell impedance in the first 2–3 h, followed by a steady second phase (1–2 h), and a third phase represented by a further increase with higher values than those observed during the first phases (Fig. 1B). These microglial impedance measurements confirmed the observations that 34% of cells exhibit an "amoeboid-shape" within the first 3 h of the LPS challenge, while within 48 h of LPS exposure 90% of the cells revealed this amoebic phenotype (Beck et al., 2008). However, the LPS-dependent Ca^{2+} alterations are in sharp contrast to the microglial morphological shapes. In sub-cultured microglia, LPS induced a strong elevation in $[Ca^{2+}]_i$ after 2 h, followed by a transient undershoot lasting for at least 20 h, before returning to basic levels detected before LPS application. Our findings are consistent with the data on LPS-induced $[Ca^{2+}]_i$ alterations previously reported by Beck et al. (2008). They reported that LPS mediated drastic calcium increases within first 2 h, although with a delay of 40–50 min, while the potassium currents were altered only after 3–6 h LPS exposure. These studies corroborate well with our finding that *KCNN*/SK3/K_{Ca}2.3 channel activation can elicit effects on microglial activation (Fig. 1G) after at least 6–8 h following LPS application. The remaining question of how calcium dysregulation was involved in microglial activation processes prompted us to investigate calcium signals in response to LPS challenge in the absence of extracellular calcium. The first increase of $[Ca^{2+}]_i$ in response to LPS is essential to promote an increase in cell impedance, since removal of extracellular calcium abolishes both the $[Ca^{2+}]_i$ increases and the second elevation of CI (Fig. 1B). *KCNN*/SK/K_{Ca}2 channel activation attenuated the initial calcium increase following 2 h of LPS treatment and also reduces the LPS-induced microglial activation after 8–30 h. Although the calcium signals are below basal levels following 4 h LPS challenge, TNF-α cytokine release is initiated in response to LPS. Cytokine release is gradually augmented during 20 h LPS treatment, while the $[Ca^{2+}]_{i,}$ was found below basal levels. The second rise in $[Ca^{2+}]_{i,}$ levels detected following 30 h LPS challenge might be due to LPS-mediated sustained cytokine release, which in turn, can trigger calcium increases, acting as a posi-

tive feedback loop mechanism. These findings imply that the initial LPS-mediated increase in $[Ca^{2+}]_i$ signals is essential for triggering delayed cytokine production (20–30 h following LPS challenge), since activation of *KCNN*/SK/K_{Ca}2 channels suppressed the initial $[Ca^{2+}]_i$ increase and also the delayed cytokine and NO release. The reduction of basal $[Ca^{2+}]_i$ signals following 4 h of LPS application might be due to the decline in amplitude of I_{DRK} and I_{IRK} currents, which would favor a more depolarized membrane potential and decreased driving force for Ca^{2+} into the cell (Beck et al., 2008). How *KCNN*/SK/K_{Ca}2 current activity is altered upon LPS challenge remains to be investigated. One hypothesis involves close association between K_{Ca}2 channels and purinergic receptors, since LPS injection into striatum markedly increased the expression of ionotropic ATP-gated purinergic P2X(7) receptors in microglia (Choi et al., 2007), and P2X(7)R receptors potentiated $[Ca^{2+}]_i$ mobilization which in turn activated *KCNN3*/SK3/K_{Ca}2.3 channels (Jelassi et al., 2011). Thus, activation of *KCNN3*/SK3/K_{Ca}2.3 channels might act in a feedback loop manner to reduce the ongoing microglial proliferation induced by ATP-sensitive receptors. In a recent study, contribution of *KCNN3*/SK3/K_{Ca}2.3 channels to microglial activation processes was discriminated using subtractive pharmacological approach based on affinities of apamin and tamapin for cloned *KCNN*/SK/K_{Ca}2 channels (Pedarzani and Stocker, 2008). The IC_{50} values for apamin are 0.7–12 nM in recombinant human *KCNN1*/SK1/K_{Ca}2.1 channels, 27–140 pM for *KCNN2*/SK2/K_{Ca}2.2 channels, and 0.6–4.0 nM for *KCNN3*/SK3/K_{Ca}2.3 channels. The K_d values for tamapin are 24 pM for *KCNN2*/SK2/K_{Ca}2.2 channels and 1.7 nM for *KCNN3*/SK3/K_{Ca}2.3 channels (Pedarzani et al., 2002). A role for *KCNN3*/SK3/K_{Ca}2.3 channels was deduced when the cellular microglial functions were inhibited by 100 nM apamin and 5 nM tamapin, but not by 250 pM tamapin (Schlichter et al., 2010). Thus, using this subtractive pharmacological approach, the authors suggested that inhibition of *KCNN3*/SK3/K_{Ca}2.3 channels reduced the neurotoxic effects of activated microglia (Schlichter et al., 2010). However, the authors did not investigate the effects of toxins on cytokine production in microglial cells. In sharp contrast with these findings, it was very recently demonstrated that riluzole activated *KCNN3*/4/K_{Ca}2.3/K_{Ca}3.1 channels thereby inducing anti-inflammatory effects in a microglial cell line (Liu et al., 2012). In this recent study, riluzole induced an increase in *KCNN3*/4/K_{Ca}2.3/K_{Ca}3.1 activity and it reduced LPS-induced proliferation and cytokine release in primary rat microglia. These findings are clearly in line with our current results showing that K_{Ca}2.3 channel activation reduces LPS-dependent microglial activation. In addition, we show here in primary microglia that apamin mitigated the effect of CyPPA on cytokine suppression, and it also reduced the effect of CyPPA on microglial proliferation as assessed by impedance measurements. To overcome the possible limitations attributed to subtractive pharmacology and to unspecific pharmacological inhibition of all *KCNN*/SK/K_{Ca}2 subtypes by apamin, we applied specific inhibitory peptides for the different *KCNN*/SK/K_{Ca}2 channel subtypes (Tuteja et al., 2010). Using these inhibitory peptides, we revealed a differential regulation of cytokine release activities only through *KCNN3*/SK3/K_{Ca}2.3 channel activation. In fact, only inhibition of *KCNN3*/SK3/K_{Ca}2.3 channel activity delayed CyPPA-dependent CI decrease for at least 15–20 h, demonstrating that in microglial cells activation of *KCNN3*/SK3/K_{Ca}2.3 channels inhibits microglial proliferation. Further, CyPPA-mediated suppression of LPS-induced IL-6 release was completely abolished by specific inhibitory peptides for *KCNN3*/SK3/K_{Ca}2.3 channels.

Major executive features of microglial activation processes, such as production and release of cytokines and chemokines, depend on *de novo* synthesis of these proteins. Since intracellular Ca^{2+} mobilization and increased $[Ca^{2+}]_i$ levels are just permissive and not initiating for cytokine production (Hoffmann et al., 2003), our study now implies that *KCNN3*/SK3/K_{Ca}2.3 channels regulate microglial $[Ca^{2+}]_i$, and, in addition, exert a differential control over cytokine production and release activities. In LPS-stimulated microglia, activation of *KCNN3*/SK3/K_{Ca}2.3 channels attenuated IL-6 release in a similar fashion as EDTA suggesting that IL-6 production and release dependence on extracellular calcium was sensitive to inhibitory modulation by *KCNN3*/SK3/K_{Ca}2.3 channels. In stimulated microglia, TNF-α release is not affected by extracellular calcium chelation, which is in sharp contrast to *KCNN3*/SK3/K_{Ca}2.3 channel activation by CyPPA that attenuated microglial activated-dependent TNF-α release. Indeed, such differential cytokine induction and kinetics were also reported in murine peritoneal macrophages or in human skeletal myocytes in response to various stimuli such as LPS, interferon (IFN)-gamma, platelet activating factor, or calcineurin (Keller et al., 2006; Marriott et al., 1998). Thus, regulation of cytokine induction and release apparently involves more than one single calcium-dependent pathway (Marriott et al., 1998).

Several studies showed that potassium channels other than *KCNN*/SK/K_{Ca}2 channels can be involved in microglial activation. *KCNN4*/K_{Ca}3.1 channels are highly expressed in microglial cells (Khanna et al., 2001) and are involved in the production of reactive oxygen species, NO, and protein tyrosine nitration (Skaper, 2011). Beneficial effects of *KCNN4*/K_{Ca}3.1 channel inhibition are mainly attributed to the selective blocker triarylmethane-34 (Wulff et al., 2000) which reduces the neurotoxicity mediated by LPS-activated microglia by reducing the expression of inducible NO synthase and caspase 3 (Kaushal et al., 2007). However, the functional difference between *KCNN4*/K_{Ca}3.1 and *KCNN3*/SK3/K_{Ca}2.3 channels remains to be further evaluated. On the contrary, diazoxide, an activator of ATP-sensitive potassium (K_{ATP}) channels, restored microglial activation in the corpus callosum after permanent bilateral occlusion of the common carotid arteries in rats (Farkas et al., 2005). Our data demonstrate that activation of *KCNN3*/SK3/K_{Ca}2.3 channels prevents characteristic features of microglial activation, such as transformation into macro-

phages, NO production, and cytokine release activities. Activation of *KCNN2/3*/K_{Ca}2.2/K_{Ca}2.3 channels by CyPPA did not alter cytokine production under control conditions, but clearly reduced LPS-induced cytokine release, which indicated a potential for CyPPA to provide protective functions by counteracting LPS-related inflammatory responses.

Our study demonstrates that extracellular calcium contributes to LPS-induced microglial proliferation and IL-6 release and by activation of *KCNN3*/SK3/K_{Ca}2.3 channels the microglial proliferation and cytokine release is strongly attenuated. Our data further suggest that *KCNN3*/SK3/K_{Ca}2.3 channels regulate microglial-dependent acute inflammatory processes, since our treatment protocol spanned over a period of 24 h. Interestingly, CyPPA applied following the onset of microglial activation at the point of LPS-induced maximum proliferation still led to a decrease of the cell index, suggesting that *KCNN3*/SK3/K_{Ca}2.3 channel activation can modulate ongoing microglial-dependent activation pathways. Moreover, activation of *KCNN*/SK/K_{Ca}2 channels elicits a dual action in the nervous system, by counteracting the effects of microglial activation as shown in this study, and by maintaining calcium homeostasis after NMDAR activation, thus preventing *in vitro* excitotoxic neuronal death and *in vivo* middle cerebral artery occlusion acute brain damage as shown in earlier studies (Allen et al., 2011; Dolga et al., 2008, 2011)

In conclusion, our data demonstrate that modulation of *KCNN3*/SK3/K_{Ca}2.3 channels may serve as a therapeutic target for reducing microglial activity and related detrimental inflammatory effects in the nervous system.

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